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14. ABSTRACT

Here we identify a novel role for PDGFR-alpha as an essential downstream target of the transcription factor Twist1 for metastasis and local invasion. Twist1 induces the formation of structures called invadopodia that concentrate protease activity to areas of the cell in contact with the extracellular matrix. Twist1-induced invasion and metastasis are dependent on invadopodia formation, as knockdown of an essential invadopodia protein, Tks5, reduces invasion and metastasis. Twist1 induces invadopodia formation by directly inducing transcription of the tyrosine kinase receptor PDGFR-alpha. Upregulation of PDGFR-alpha is necessary for an increase in Src activation observed upon expression of Twist1 in human mammary epithelial cells. Knockdown of PDGFR-alpha reduces both in vitro and in vivo invasion and metastasis. In addition, we identify Twist1 and PDGFR-alpha as markers of poor survival in breast cancer patient populations.

15. SUBJECT TERMS
Twist1, PDGFR, invadopodia, metastasis, invasion, Src, ADAM12

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Table of Contents

Introduction

Distant metastases are responsible for the vast majority of breast cancer deaths. This process involves several discrete steps beginning with local invasion of nearby tissues by carcinoma cells followed by intravasation into capillaries and dissemination through the bloodstream. Extravasation from the circulatory system through the endothelium may occur in distant organs and tissues thereby establishing micrometastases which may proliferate and give rise to secondary tumors (Kalluri and Robert A Weinberg 2009). Acquiring the ability to migrate and invade is therefore essential to the metastatic process.

The initial steps of breast cancer metastasis, local invasion and intravasation, require carcinoma cells to lose cell-cell adhesion and gain the ability to migrate and invade (Hanahan and R A Weinberg 2000). This process recapitulates several key steps in embryonic development including neural crest cell migration. This phenomenon, occurring in both cancer cells and during embryonic development, is the highly-conserved Epithelial-Mesenchymal Transition (EMT). In many breast cancers, this same EMT process has been implicated in the progression of the primary tumor to metastatic disease (Yang et al. 2004). Biochemically, EMT is associated with a loss of epithelial adhesion proteins such as E-cadherin and α-catenin and a gain of mesenchymal cell markers such as vimentin and fibronectin (Lee et al. 2006). This drastic change in cell characteristics associates well with increased carcinoma cell motility and invasion.

Previous studies in our laboratory have demonstrated that expression of the basic Helix-Loop-Helix (bHLH) transcription factor Twist1 is essential for the ability of breast tumor cells to metastasize from the mammary gland to the lung in a mouse breast tumor model (Yang et al. 2004). Twist1 regulates the loss of E-cadherin mediated junctions during EMT by regulating the expression of the E-cadherin repressor Slug (Casas et al. 2011). Loss of cadherin-mediated junctions is a passive process, however, that is necessary but not sufficient for metastasis (Onder et al. 2008). We therefore investigated if Twist1 actively induces local invasion and metastasis via the formation of structures called invadopodia.

Invadopodia are actin-rich protrusions on the basal surface of cells that concentrate protease activity to areas of the cell in contact with the extracellular matrix (ECM) (Linder 2009). Src kinase activation promotes formation of invadopodia by phosphorylating a variety of structural proteins, including cortactin and Tks5 (Seals et al. 2005; Webb, Eves, and Mak 2006). Invadopodia can be visualized by staining for F-actin and cortactin and their activity quantified by seeding the cells on fluorescein isothiocyante (FITC) labeled gelatin (Bowden et al. 2006). The invadopodia will degrade the fluorescent gelatin, leaving behind non-fluorescent holes that can be quantified (see Figure 1C for an example image).

We found that Twist1 induced robust formation of invadopodia when overexpressed in human mammary epithelial cells (HMLEs) when stained for cortactin and F-actin (Figure 1A-B). In addition, these invadopodia were active and able to degrade ECM (Figure 1C-D). We observed similar results upon assaying for invadopodia formation and function in cell lines endogenously expressing Twist1. Invadopodia formation in these cells was dependent on Twist1, as knockdown of Twist1 significantly reduced invadopodia formation and function (see Appendix, Eckert et al. 2011).

My work over the last year has been to elucidate the role of the downstream targets of Twist1 in regulating invadopodia formation. I identified PDGFRα as a necessary downstream target of Twist1 required for invadopodia formation and metastasis. For additional details not covered in this report, please see the attached manuscript (Eckert et al. 2011).

Body

Aim 1: Identify the transcriptional targets of Twist1 responsible for invadopodia formation.

Subaim 1A: Identify components of invadopodia upregulated in response to Twist1 at the RNA and protein levels.

We found that Twist1 did not significantly induce the expression of structural components of invadopodia (Tks5 or cortactin) and the overall level of proteases (MMP2, MMP9, and MT1-MMP) were not dramatically altered upon exogenous Twist1 expression in HMLEs. We did, however, observe a robust and significant upregulation of PDGFRα (Figure 2A). This was significant, as PDGFRs are directly upstream of Src activity, a necessary step in invadopodia biogenesis (Kypta et al. 1990). We also observed that upon expression of Twist1 in HMLEs, PDGFRα was phosphorylated, corresponding to its activation, and Src activity was increased (Figure 2A and B). We therefore hypothesized that PDGFRα was responsible for the induction of Src activation during Twist1-driven EMT.

We have also begun preliminary experiments investigating the role of ADAM12 in Twist1-driven invadopodia formation. ADAM12 is dramatically induced at the RNA and protein level. This is significant, as ADAM12 is known to not only bind to the important invadopodia component protein Tks5, but also to be localized to invadopodia (Seals et al. 2005). Ongoing experiments in our lab are further characterizing the role of ADAM12, while we focused our research on the role of PDGFRα.

Subaim 1B: Investigate if candidate proteins identified in Subaim 1A are necessary for invadopodia formation via knockdown with shRNAs and administration of protease inhibitors.

We were able to generate multiple knockdowns of PDGFRα in HMLE-Twist1 cells that significantly reduced the expression of PDGFRα at the RNA and protein level (Figure 3A). In addition, knockdown of PDGFRα significantly reduced gelatin degradation in HMLE cells overexpressing Twist1 (Figure 3C). In lieu of protease inhibitors, HMLE-Twist1 cells were also treated with a PDGFRα blocking antibody that was also able to significantly reduce invadopodia formation (as assayed with F-actin/cortactin costaining) and gelatin degradation (Figure 3B and C)

Experiments regarding the role of ADAM12 in invadopodia are ongoing. Preliminary data using shRNAs against ADAM12 suggest that ADAM12 plays a role in degradation, but not initial formation of invadopodia. We are currently performing rescue and inhibition experiments to determine if the intrinsic protease activity of ADAM12 or other domains are necessary for its role in invadopodia-mediated degradation. Experimental results are still extremely preliminary at this time.

Aim 2: Determine if invadopodia are responsible for Twist1-induced breast cancer cell invasion and metastasis.

Subaim 2A: Determine if invadopodia are necessary for Twist1-induced invadopodia activity with gelatin and ECM degradation assays.

As we identified PDGFRα as a central regulator of invadopodia formation during Twist1-induced EMT, we focused on the role of the kinase activity of PDGFRα and its downstream targets in mediating Twist1-induced invasion and matrix degradation. As mentioned above, knockdown and inhibition of PDGFRα significantly reduced gelatin degradation (Figure 3A-C). In addition, knockdown of PDGFRα was also able to reduce invasion through Matrigel (see Appendix, Eckert et al. 2011). To determine if the gelatin degradation and invasion observed upon Twist1 expression was truly invadopodia-driven, we also knocked down Tks5, a structural

component of invadopodia with no other known roles. Upon knockdown of Tks5 in HMLE-Twist1 cells, we observed significantly decreased gelatin degradation and a virtual elimination of invadopodia formation, suggesting that Twist1-induced invasion and matrix degradation were invadopodia-driven (Figure 4A and B).

Subaim 2B: Determine if Twist1-induced invadopodia affect breast cancer metastasis with a subcutaneous mouse tumor implantation model of breast cancer metastasis.

HMLE-Twist1 cells expressing shRNAs against Tks5 or PDGFRα were infected with GFP and Ras to allow tracking in vivo and to transform them, respectively. Knockdown of Tks5 was used to determine if invadopodia were required for metastasis, as PDGFRα could presumably play other roles in vascularization or tumor growth. These cells were injected subcutaneously in nude mice and allowed to grow to 2 cm in diameter. No significant differences in tumor growth in vivo was observed. We did, however, observed a significant decrease in both metastasis to the lung and local invasion (Figure 4C and D and see Appendix, Eckert et al. 2011). This suggested that Twist1-mediated metastasis is both PDGFRα and invadopodia dependent. In addition, we found that Twist1 and PDGFRα were highly negatively correlated with survival in human breast cancer patient tissue specimens (See Appendix, Eckert et al. 2011).

Aim 3: Characterize the mechanism of Twist1 induction of invadopodia.

Subaim 3A: Determine if candidate proteins identified in Aim1 are direct targets of Twist1 with promoter analysis including use of bioinformatics and luciferase promoter assays.

We cloned the human PDGFRα promoter, which contained an E-box element (CANNTG), and inserted it into a luciferase-reporter plasmid. Since Twist1-mediated transcription is regulated by binding to E-box elements, we also mutated the E-box to be nonfunctional. When transfected into cells with Twist1 and the Twist1 partner E12, we observed a significant increase in luciferase activity that was attenuated upon mutation of the E-box (Figure 5). This suggested that PDGFRα is a direct transcriptional target of Twist1.

Subaim 3B: Determine relevant pathways involved in regulating invadopodia activity via post-translational modification and protein interactions by assaying activation of specific pathways.

As mentioned above, we observed a significant increase in Src activation upon Twist1 expression in HMLE cells (Figure 2B). Inhibition of both PDGFRα (Figure 3A-C) or Src activity (see Appendix, Eckert et al. 2011) significantly reduced invadopodia formation. This concurs with previous data highlighting the central role of Src activation in regulating and inducing invadopodia formation. In addition, treatment with the broad-spectrum metalloprotease inhibitor GM6001 also eliminated gelatin degradation, indicating that Twist1-induced invadopodia are metalloprotease dependent.

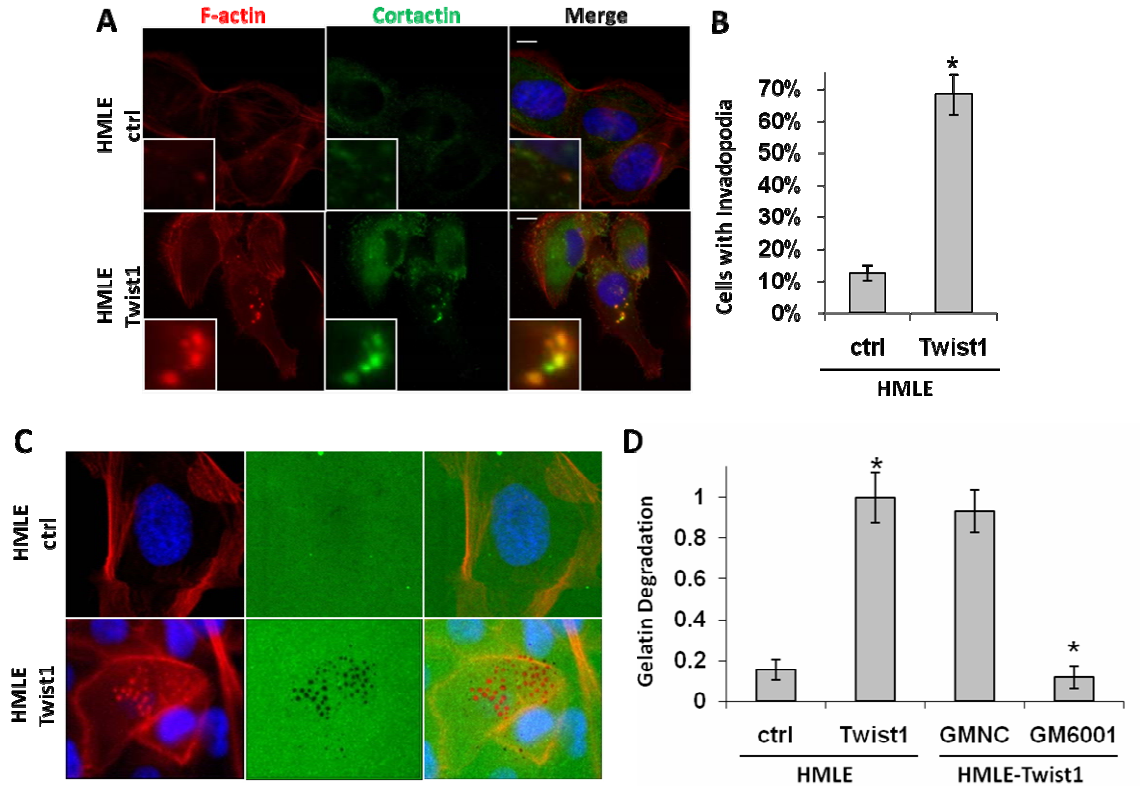

Figure 1: **(A)** Twist1 induces invadopodia formation in HMLE cells overexpressing Twist1, when stained for the invadopodia markers cortactin and F-actin. **(B)** There is a significant increase in the number of cells quantified as positive for invadopodia (at least one invadopodia per cell) **(C)** Twist1 overexpression increases gelatin degradation (black area) in HMEC cells **(D)** The increase in gelatin degradation is significant, and can be reduced when treated with the metalloprotease inhibitor GM6001 (GMNC = GM6001 negative control compound). Error bars are SEM. *p< 0.05, student's t-test.

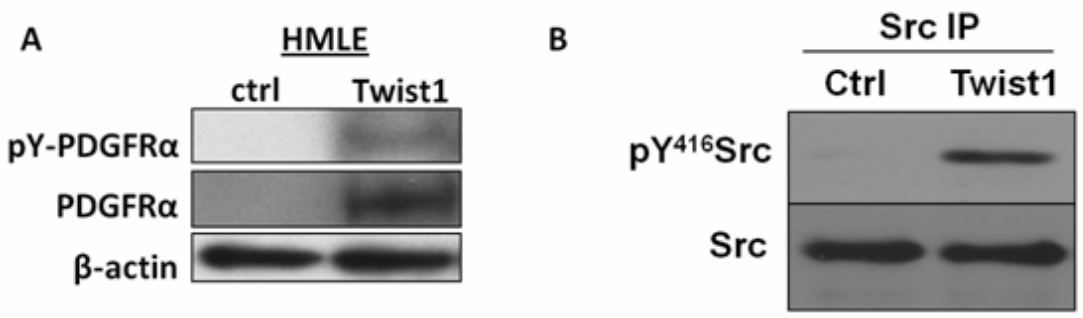

Figure 2: **(A)** Immunoblot for total and phosphorylated PDGFRα; a robust induction of both total and activated PDGFRα can be observed in the cells over expressing Twist1. **(B)** Src was immunoprecipitated from HMLE cells expressing control or Twist1 overexpression vectors and blotted for total Src and tyrosine phosphorylated Src. A dramatic increase in Src phosphorylation is observed upon expression of Twist1.

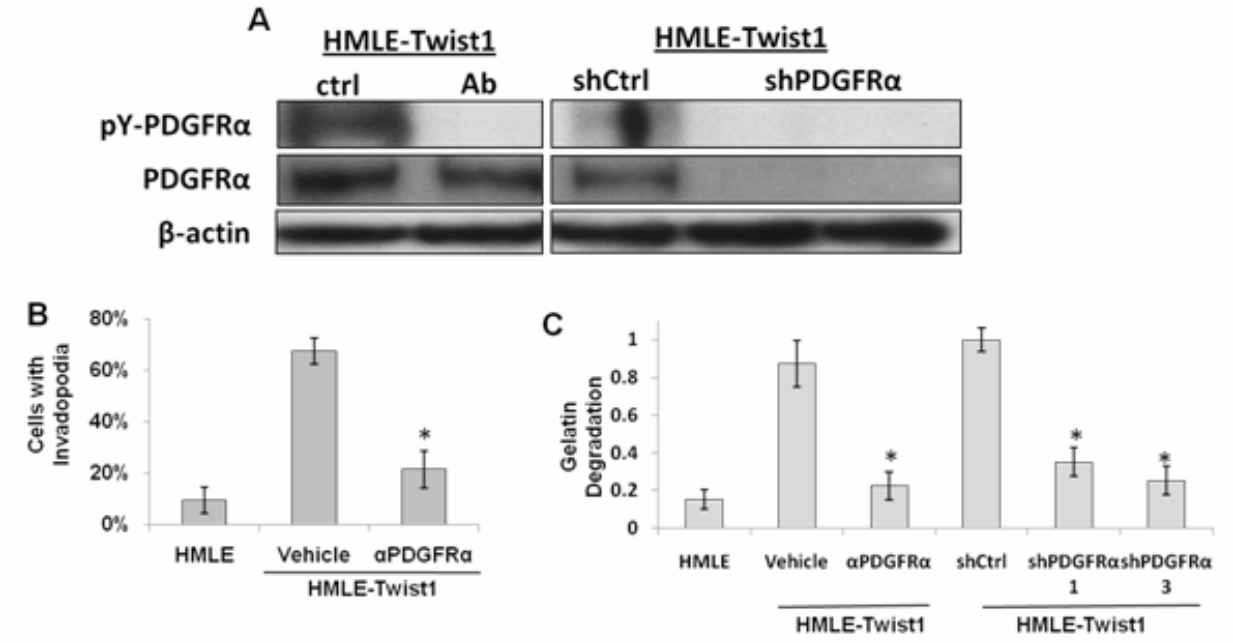

Figure 3: (A) Immunoblot for total and phosphorylated PDGFRα; knockdown of PDGFRα reduces total protein levels while inhibition with a monoclonal blocking antibody (Ab) reduces activation of PDGFRα. **(B)** Quantification of cells with invadopodia upon treatment with a monoclonal blocking antibody (αPDGFR α); a significant decrease in cells with invadopodia can be observed following treatment. **(C)** Quantification of gelatin degradation. Both knockdown of PDGFR α or inhibition with a monoclonal blocking antibody (αPDGFR α) significantly decreases gelatin degradation. Error bars are SEM. *p<0.05.

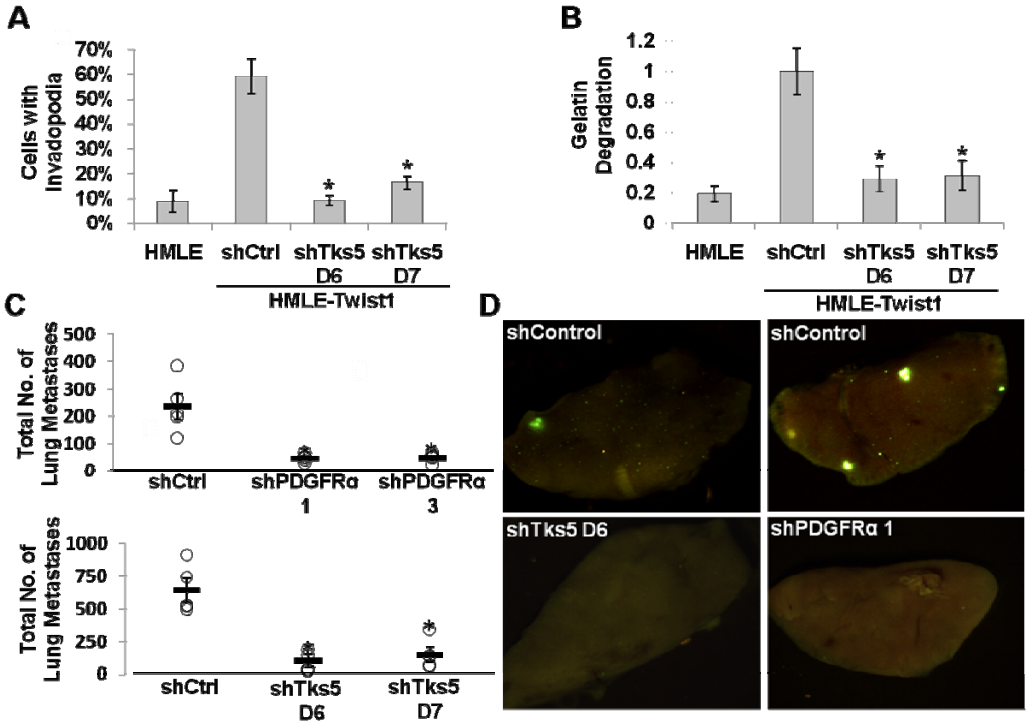

Figure 4: (A-B) Quantification of invadopodia formation (A) and gelatin degradation in HMLE-Twist1 cells expressing control or Tks5 knockdown shRNAs; both knockdowns significantly reduce both gelatin degradation and invadopodia formation. **(C)** Quantification of number of GFP-positive puncta observed in the lungs of mice injected with HMLE-Twist1 cells expressing Ras and GFP; both knockdown of PDGFRα and Tks5 significantly reduce metastases **(D)** Representative images of lungs from Mice injected with HMLE-Twist1 cells expressing the indicated shRNAs. Metastases appear as green puncta. Error bars are SEM. *p< 0.05.

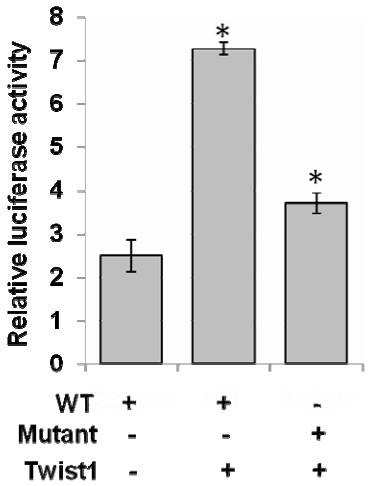

Figure 5: Relative luciferase activity of 293T cells transfected with either WT PDGFRα promoter plasmid, WT PDGFR α promoter plasmid and Twist1, or with a mutated PDGFR α promoter plasmid lacking an E-box (Mutant) and Twist1. A significant increase in luciferase activity is observed with the WT plasmid, while mutation of the E-box significantly decreases luciferase activity. Error bars are SEM. *p< 0.05.

Key Research Accomplishments

- Twist1-induced invasion and metastasis are invadopodia-dependent
- Twist1 directly regulates expression of PDGFRα
- Twist1 increases Src activity via upregulation of PDGFRα
- PDGFRα is necessary for Twist1-mediated invadopodia formation and gelatin degradation
- PDGFRα and invadopodia are necessary for Twist1-induced metastasis
- Training in cell biology techniques (immunofluorescence microscopy, *in situ* gelatin zymography, virus production and infection, Matrigel invasion assays)
- Training in biochemical techniques (immunoprecipitation, Western blotting, luciferase assays)
- Training in animal models of cancer (subcutaneous tumor implantation model of metastasis)
- Opportunities to present research at weekly lab meetings and departmental seminars

Reportable Outcomes

Manuscripts published from work supported by this grant:

Eckert MA, Lwin TM, Chang AT, Kim J, Danis E, Ohno-Machado L, Yang J. Twist1-induced invadopodia formation promotes tumor metastasis. *Cancer Cell*. 2011 Mar 8;19(3):372-86.

(see Appendix, Eckert et al. 2011)

Successfully applied to the Howard Hughes Medical Institute's Med-into-Grad program and associated funding at UCSD for the 2011-2012 academic year.

Conclusions

Over the past year, I have identified a novel role for PDGFRα as a downstream target of Twist1 responsible for inducing both local invasion and metastasis. This is the first identification of a transcriptional regulator of invadopodia formation and the first time a specific transcription factor has been identified as a regulator of PDGFRα expression. The identification of invadopodia as a vital downstream target of Twist1 in EMT reveals several new targets for inhibition of metastasis in cells undergoing EMT. Inhibition of not only PDGFRα, but also Src or metalloproteases, could inhibit metastases in these cells. Our identification of PDGFRα and Twist1 as markers of poor prognosis in breast cancer patient populations could serve as a set of markers for targeted treatments. I have also had the opportunity to not only gain experience in research, both designing and performing experiments, but also had the opportunity to gain experience in preparing a manuscript and plan to present my work at meetings this autumn.

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Cancer Cell
Article

Twist1-Induced Invadopodia Formation Promotes Tumor Metastasis

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SUMMARY

The Twist1 transcription factor is known to promote tumor metastasis and induce Epithelial-Mesenchymal Transition (EMT). Here, we report that Twist1 is capable of promoting the formation of invadopodia, specialized membrane protrusions for extracellular matrix degradation. Twist1 induces PDGFRα expression, which in turn activates Src, to promote invadopodia formation. We show that Twist1 and PDGFRα are central mediators of invadopodia formation in response to various EMT-inducing signals. Induction of PDGFRα and invadopodia is essential for Twist1 to promote tumor metastasis. Consistent with PDGFRα being a direct transcriptional target of Twist1, coexpression of Twist1 and PDGFRα predicts poor survival in breast tumor patients. Therefore, invadopodia-mediated matrix degradation is a key function of Twist1 in promoting tumor metastasis.

INTRODUCTION

During metastasis, carcinoma cells acquire the ability to invade surrounding tissues and intravasate through the endothelium to enter systemic circulation. Both the invasion and intravasation processes require degradation of basement membrane and extracellular matrix (ECM). Although proteolytic activity is associated with increased metastasis and poor clinical outcome, the molecular triggers for matrix degradation in tumor cells are largely unknown.

Invadopodia are specialized actin-based membrane protrusions found in cancer cells that degrade ECM via localization of proteases (Tarone et al., 1985, Chen, 1989). Their ability to mediate focal ECM degradation suggests a critical role for invadopodia in tumor invasion and metastasis. However, a definitive role for invadopodia in local invasion and metastasis in vivo has not yet been clearly demonstrated. As actin-based structures, invadopodia contain a primarily branched F-actin core and actin regulatory proteins, such as cortactin, WASp, and the Arp2/3 complex (Linder, 2007). The SH3 domain-rich proteins Tks4 (Buschman et al., 2009) and Tks5 (Seals et al., 2005) function as essential adaptor proteins in clustering structural and enzymatic components of invadopodia. The matrix degradation activity of invadopodia has been associated with a large number of proteases, including membrane type MMPs (MT1-MMP) (Linder, 2007). Invadopodia formation requires tyrosine phosphorylation of several invadopodia components including cortactin (Ayala et al., 2008), Tks4 (Buschman et al., 2009), and Tks5 (Seals et al., 2005) by Src family kinases.

Our previous study found that the Twist1 transcription factor, a key regulator of early embryonic morphogenesis, was essential for the ability of tumor cells to metastasize from the mammary

Significance

Studies suggest that the EMT-inducing transcription factors play critical roles in tumor metastasis. A major question is what are the cellular functions and transcriptional targets of individual EMT-inducing transcription factors required for tumor metastasis. Our study identifies a unique function of Twist1 in promoting invadopodia-mediated matrix degradation, which is essential for its ability to promote metastasis. Formation of invadopodia and loss of cell adhesion are regulated by different transcription factors. This explains why multiple factors need to be activated coordinately to promote carcinoma cells to undergo EMT and invade. We also identify PDGFRα as a direct transcriptional target of Twist1 in promoting invadopodia formation and tumor metastasis, therefore suggesting that PDGFRs might be potential targets for anti-metastasis therapies.

gland to the lung in a mouse breast tumor model and was highly expressed in invasive human lobular breast cancer (Yang et al., 2004). Since then, studies have also associated Twist1 expression with many aggressive human cancers, such as melanomas, neuroblastomas, prostate cancers, and gastric cancers (Peinado et al., 2007). Twist1 can activate a latent developmental program termed the epithelial-mesenchymal transition (EMT), thus enabling carcinoma cells to dissociate from each other and migrate.

The EMT program is a highly conserved developmental program that promotes epithelial cell dissociation and migration to different sites during embryogenesis. During EMT, cells lose their epithelial characteristics, including cell adhesion and polarity, and acquire a mesenchymal morphology and the ability to migrate (Hay, 1995). Biochemically, cells downregulate epithelial markers such as adherens junction proteins E-cadherin and catenins and express mesenchymal markers including vimentin and fibronectin (Boyer and Thiery, 1993). In addition to Twist1, the zinc-finger transcription factors, including Snail, Slug, ZEB1, and ZEB2 (Peinado et al., 2007), can also activate the EMT program by directly binding the E-boxes of the E-cadherin promoter to suppress its transcription. However, it is unclear how Twist1, as a bHLH transcription factor, controls the EMT program. In this study, we test the hypothesis that Twist1 plays a major role in regulating ECM degradation to promote tumor metastasis.

RESULTS

Twist1 Is Necessary and Sufficient for Invadopodia Formation and Function

Our previous studies found that Twist1 expression was associated with increased metastatic potentials in a series of mouse mammary tumor cell lines, including 67NR, 168FARN, and 4T1 (Yang et al., 2004). Furthermore, Twist1 is required for the ability of 4T1 cells to metastasize from the mammary gland to the lung. To dissect the cellular functions of Twist1 in promoting tumor metastasis, we first tested whether expression of Twist1 was associated with increased ability to degrade ECM. 67NR, 168FARN, and 4T1 cells were plated onto FITC-conjugated gelatin matrix to assess their abilities to degrade matrix. We found that Twist1-expressing metastatic 168FARN and 4T1 cells potently degraded ECM in 8 hr, while nonmetastatic 67NR cells that do not express Twist1 failed to do so (Figures 1A–1C). To test whether Twist1 is required for the ability of 168FARN and 4T1 cells to degrade ECM, 168FARN and 4T1 cells expressing two independent shRNAs against Twist1 were processed for the matrix degradation assay (Figure 1A). Indeed, we found that suppressing Twist1 expression resulted in a potent reduction in matrix degradation in both cell types (Figures 1B and 1C). Together, these results demonstrate that Twist1 is required for ECM degradation ability in tumor cells.

Localized matrix degradation can be mediated through actin-based subcellular protrusions called invadopodia. Colocalization of F-actin with the actin-bundling protein cortactin (Bowden et al., 2006) or the unique adaptor protein Tks5 (Abram et al., 2003) can be used to identify invadopodia. To determine whether invadopodia are present in 168FARN and 4T1 cells and whether Twist1 is required for invadopodia formation, we examined the presence of invadopodia in 168FARN and 4T1 cells by immunofluorescence. Invadopodia are transient structures, so only a fraction of cells possess invadopodia at any given time. Indeed, over 50% 168FARN and 4T1 cells contain invadopodia, while suppression of Twist1 expression reduced the occurrence of invadopodia to 5%–20% in both cell lines (Figures 1D–1F; see Figures S1A and S1B available online). These data indicate that Twist1 is necessary for the formation of invadopodia for ECM degradation.

Since 168FARN and 4T1 mouse tumor cells contain additional genetic and epigenetic changes essential for their tumorigenic and metastatic abilities (Mani et al., 2007), we next tested whether Twist1 is sufficient to promote invadopodia formation and matrix degradation in HMLE cells, immortalized normal human mammary epithelial cells. As reported, expression of Twist1 induced EMT in HMLE cells (Yang et al., 2004). We examined the presence of invadopodia and found that over 60% of HMLE cells expressing Twist1 contained invadopodia, compared with 10% of HMLE control cells with invadopodia (Figures 2A and 2B; Figure S2A). Importantly, these invadopodia were all localized to the basal surface of the cell directly adjacent to the underlying matrix when examined with Z-sectioning (Figure 2C). To determine whether these Twist1-induced invadopodia are functional, we compared the ability of these two cell lines to degrade matrix using the FITC-gelatin degradation assay. Expression of Twist1 increased matrix degradation by approximately 10-fold (Figures 2D and 2E). Strikingly, focal matrix degradation precisely colocalized with F-actin positive puncta (Figure 2D), indicating that Twist1 is sufficient to promote the formation of functional invadopodia in HMLE cells. Furthermore, Twist1-induced matrix degradation is protease-driven since suppression of metalloproteases by GM6001 inhibited the ability of HMLE-Twist1 cells to degrade FITC-gelatin (Figure 2E). Together, these data demonstrate that Twist1 is both necessary and sufficient to promote invadopodia formation and focal matrix degradation.

Twist1-Mediated Matrix Degradation Is Invadopodia-Driven and Src Dependent

Since both invadopodia-associated proteases and secreted proteases can mediate matrix degradation, we next set out to determine whether invadopodia, not secreted proteases, are solely responsible for Twist1-induced matrix degradation. In HMLE-Twist1 cells, we expressed shRNAs against Tks5, an adaptor protein that is required for invadopodia formation, but not MMP secretion (Seals et al., 2005). Both shRNAs effectively suppressed Tks5 expression (Figure S3A), and gelatin zymography showed that knockdown of Tks5 did not affect the secretion of proteases, mainly MMP2, into conditioned media (Figure S3B). In contrast, suppression of Tks5 significantly reduced their abilities to form invadopodia (Figures 3A and 3B) and degrade FITC-gelatin matrix (Figure 3C). Complementary to these data, Boyden chamber migration and invasion assays showed that suppression of Tks5 inhibited the ability of HMLE-Twist1 cells to invade through Matrigel, but did not affect cell migration (Figures S3C and S3D). Together, these results demonstrate that the protease activity associated with invadopodia is the sole mediator of Twist1-induced matrix degradation.

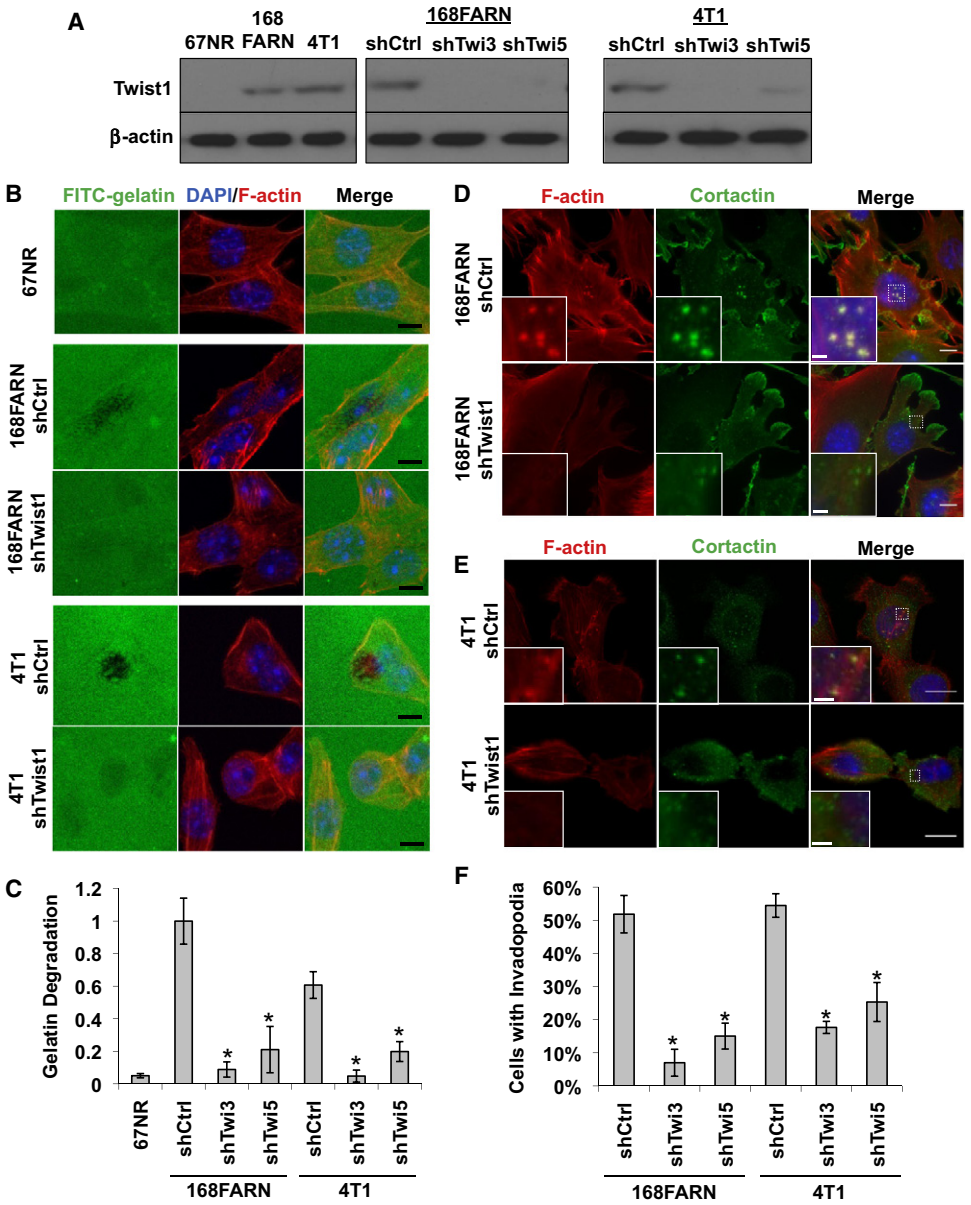

Figure 1. Twist1 Is Necessary for Invadopodia Formation

(A) Indicated cell lysates were analyzed by SDS-PAGE and probed for Twist1 and β-actin.

(B) 67NR, 168FARN (expressing control or Twist1 knockdown shRNA), and 4T1 (expressing control or Twist1 knockdown shRNA) cells were plated on FITC-conjugated gelatin (green) for 8 hr. F-actin was stained with phalloidin (red) and nuclei with DAPI (blue). Areas of gelatin degradation appear as punctuate black areas beneath the cells.

(C) Quantification of FITC-gelatin degradation. N = 150 cells/sample. *$p < 0.02$.

(D and E) 168FARN and 4T1 cells expressing control or Twist1 shRNAs were stained with phalloidin (red), DAPI (blue), and cortactin (green).

(F) Quantification of percentage of cells with invadopodia. N = 150 cells/sample. *$p < 0.02$.

Error bars are standard error of mean (SEM). Scale bars are 1 μm for insets, 5 μm for full images.

See also Figure S1.

We next set out to understand how Twist1 promotes invadopodia formation. While no transcription factor has been implicated in invadopodia regulation, tyrosine phosphorylation of invadopodia components, including cortactin and Tks5, is necessary for invadopodia formation (Ayala et al., 2008). We therefore assessed whether tyrosine phosphorylation at invadopodia was increased in HMLE-Twist1 cells. Immunofluorescence staining with a phosphotyrosine antibody revealed enrichment of phosphotyrosine at invadopodia (Figure 3D). Cortactin immunoprecipitated from HMLE-Twist1 cells also showed increased tyrosine phosphorylation compared to HMLE control cells (Figure 3E).

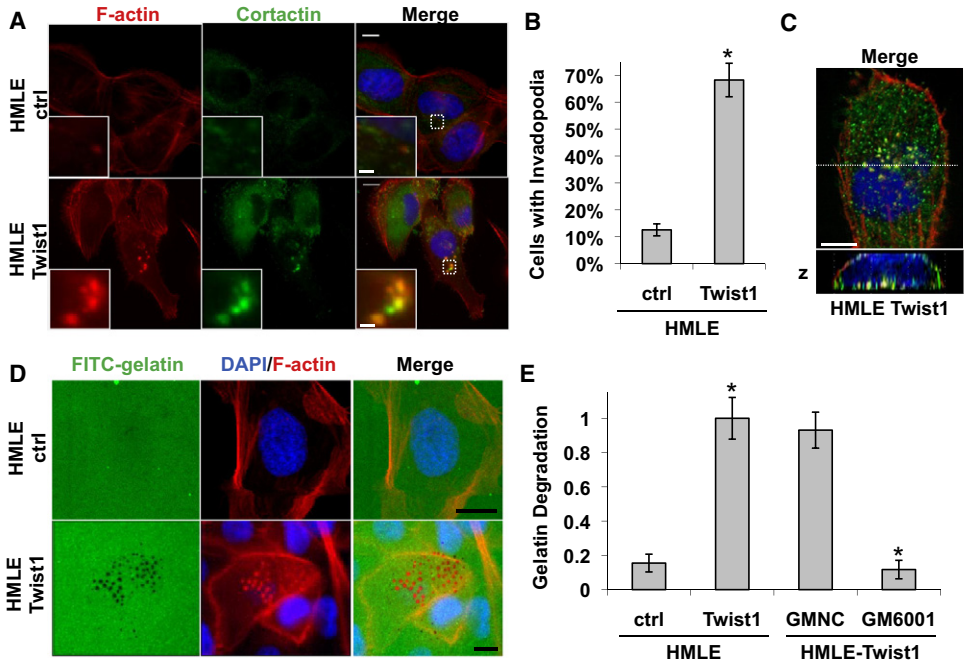

Figure 2. Twist1 Is Sufficient to Promote Invadopodia Formation

(A) HMLE cells expressing a control vector or Twist1 were plated on 0.2% gelatin matrix for 72 hr and invadopodia were visualized by colocalization of cortactin (green) and F-actin (red).

(B) Quantification of cells with invadopodia. N = 150 cells/sample. *$p < 0.02$.

(C) Colocalization of F-actin (red) and cortactin (green) is restricted to the basal side of cells in direct contact with the underlying matrix.

(D) HMLE control or HMLE-Twist1 cells were plated on FITC-gelatin for 8 hr and stained for F-actin (red) and nuclei (blue).

(E) Quantification of degradation by HMLE-ctrl and HMLE-Twist1 cells and HMLE-Twist1 cells treated with 25 μM GM6001 Negative Control (GMNC) or 25 μM GM6001 for 8 hr. N = 150 cells/sample. *$p < 0.02$.

Error bars are SEM. Scale bars are 1 μm for insets, 5 μm for full images.

See also Figure S2.

Src family kinases are the major kinases that promote tyrosine phosphorylation and formation of invadopodia. We therefore examined whether Twist1 induced expression of any of the three major Src family kinases, Src, Yes, and Fyn. Both real-time RT-PCR and immunoblotting analyses showed that none of the three Src kinases were greatly induced by Twist1 (Figures S3E and S3F; Figure 3E). Interestingly, when we probed for the activation status of Src, Yes, and Fyn in HMLE-Twist1 cells using an antibody recognizing the active form of Src family kinases (phosphotyrosine 416), Src was significantly activated upon Twist1 expression (Figure 3E), while Yes and Fyn phosphorylation remained constant (Figure S3F). These data suggest that activation of Src kinase activity, but not transcriptional induction of Src kinase expression, might be responsible for tyrosine phosphorylation at invadopodia in HMLE-Twist1 cells. To determine whether Src kinase activity is required for Twist1-induced invadopodia function, we treated HMLE-Twist1 cells with SU6656, a selective inhibitor of Src family kinases (Blake et al., 2000) (Figure S3G) or expressed a dominant-negative Src ($Src^{K295M/Y527F}$) (Figure S3H). Both treatments reduced the ability of HMLE-Twist1 cells to degrade matrix by 5-fold (Figure 3F), indicating that Src kinase activity is essential for Twist1-mediated invadopodia function. Treatment with SU6656 also inhibited colocalization of the phosphotyrosine signal with F-actin (Figure 3D) and caused a significant reduction in the number of cells that formed invadopodia (Figure 3G). Together, these results indicate that Twist1-induced invadopodia formation and function is dependent on activation of the Src kinase.

Twist1-Induced PDGFR Expression and Activation Is Required for Invadopodia Formation

As a transcription factor, Twist1 cannot directly activate Src kinase, so we probed how Twist1 promotes activation of Src in HMLE-Twist1 cells. Since activation of Src kinase is downstream of growth factor receptor (GFR) activation, we examined induction of known GFRs upstream of Src by Twist1. Using an inducible Twist1 (Twist1-ER) construct (Mani et al., 2008), we found that expression of PDGFRα mRNAs increased 3-fold within 3 hr of Twist1 activation and reached over 6000-fold induction at Day 15, while induction of PDGFRβ mRNAs occurred significantly later (Figure 4A). PDGFRs can directly activate Src family kinases by tyrosine phosphorylation (Kypta et al., 1990), and activation of a PDGF autocrine loop is associated with the EMT program (Jechlinger et al., 2003). We found that PDGFRα and β proteins were also induced in HMLE-Twist1 cells and both PDGFR α and β were phosphorylated at tyrosine residues corresponding to their active states (Figure 4B). This activation of PDGFR without exogenous PDGF ligands implies the existence of an autocrine activation loop in vitro most likely mediated by PDGF-C, the only PDGF ligand significantly expressed and

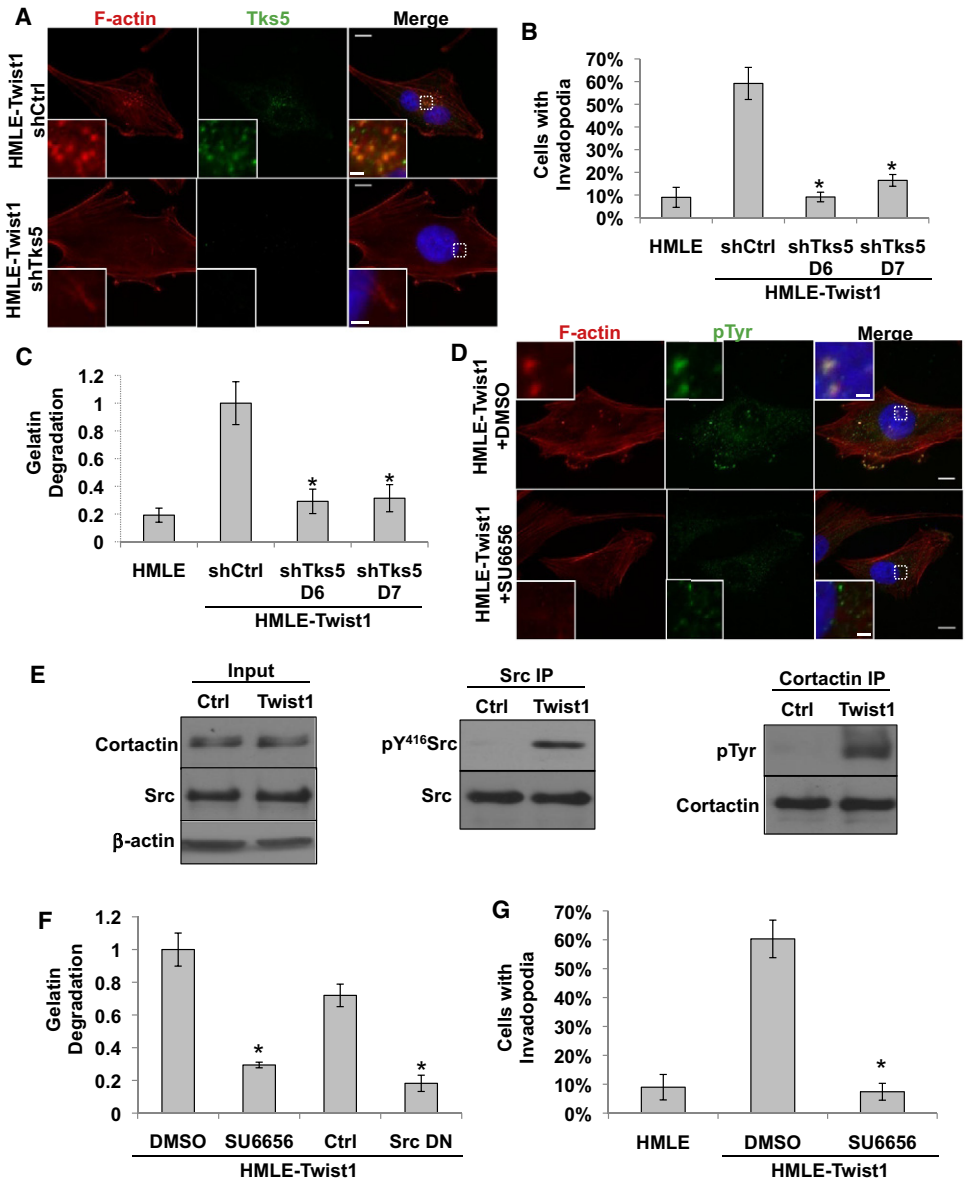

Figure 3. Twist1-Mediated Matrix Degradation Is Invadopodia Driven and Src Dependent

(A) HMLE-Twist1 cells expressing a control or Tks5 shRNA were plated on 0.2% gelatin and stained for Tks5 (green) or phosphotyrosine (green) and F-actin (red).

(B) Quantification of cells with invadopodia. N = 150 cells/sample. *$p < 0.02$.

(C) Quantification of FITC-gelatin degradation. N = 150 cells/sample. *$p < 0.02$.

(D) HMLE-Twist1 cells were plated on 0.2% gelatin and treated with treated with DMSO or 5 μM SU6656 for 12 hr and stained for phosphotyrosine (green) and F-actin (red).

(E) Cortactin and Src were immunoprecipitated from HMLE control and HMLE-Twist1 cell lysates, analyzed by SDS-PAGE, and probed for cortactin and phosphotyrosine and Src and pTyr^{416}Src, respectively. Input lysates were probed for β-actin, Src, and cortactin.

(F) Quantification of FITC-gelatin degradation. Indicated cells were treated with 5 μM SU6656 or DMSO for 12 hr or transfected with control or SrcK295M/Y527F vectors. N = 150 cells/sample. *$p < 0.02$.

(G) Quantification of cells with invadopodia. N = 150 cells/sample. *$p < 0.02$.

Error bars are SEM. Scale bars are 1 μm for insets, 5 μm for full images.

See also Figure S3.

upregulated upon activation of Twist1 in HMLE cells (Figure S4A). Upregulation of PDGFRs by Twist1 therefore presented a potential mechanism for activation of Src by Twist1.

We next set out to determine whether activation of PDGFRs is required for Twist1-induced invadopodia formation and matrix degradation. Given the immediate and robust induction of PDGFRα upon Twist1 activation, we focused on inhibiting PDGFRα to examine its role in mediating Twist1-induced Src activation and invadopodia formation. We first treated the HMLE-Twist1 cells with a monoclonal blocking antibody

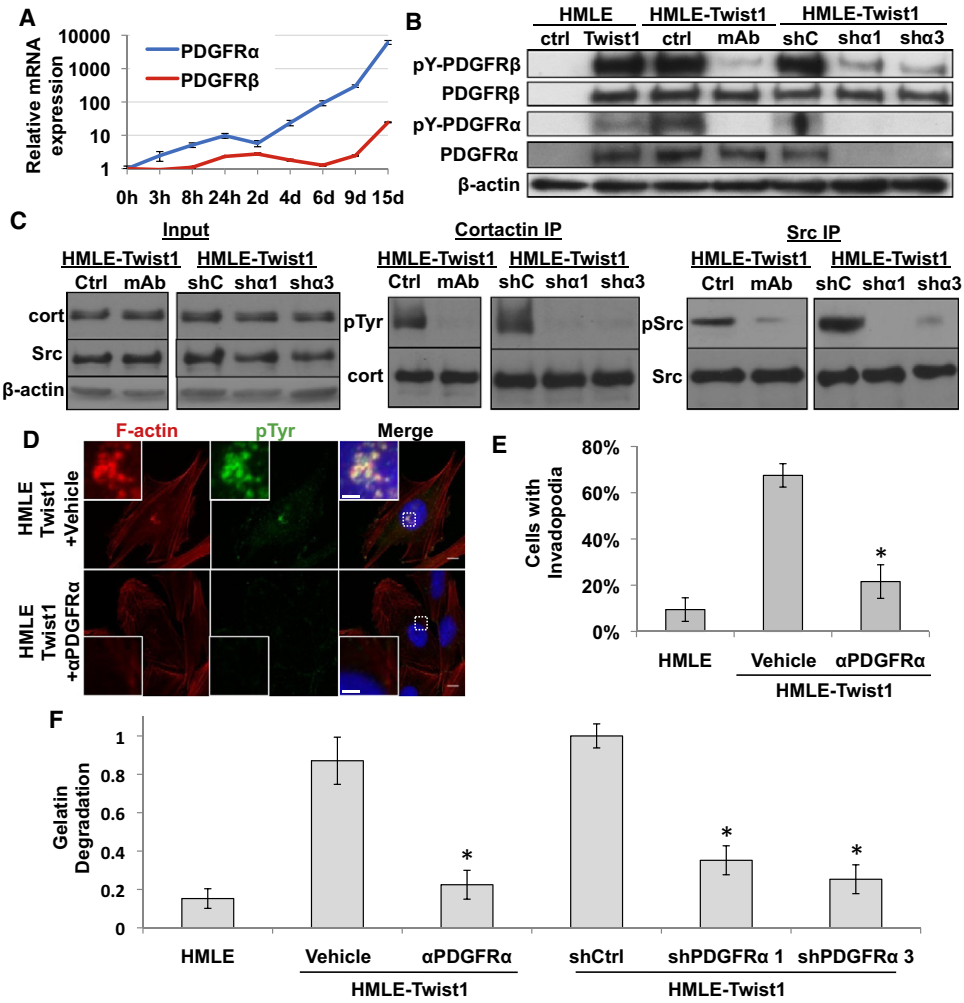

Figure 4. Twist1-Induced PDGFR Expression and Activation Is Required for Invadopodia Formation

(A) Real-time PCR analysis of PDGFRα and PDGFRβ expression in HMLE-Twist1-ER cells treated with 20 nM 4-hydroxy-tamoxifen.

(B) Cell lysates from HMLE control, HMLE-Twist1 cells, HMLE-Twist1 cells treated with vehicle or 8 μg/ml PDGFRα blocking antibody (ctrl and mAb), and HMLE-Twist1 cells expressing control (shC) or PDGFR (shα1 and 3) shRNA were analyzed by SDS-PAGE and probed for β-actin, PDGFRα, PDGFRβ, pTyr^{754}PDGFRα, and pTyr^{1009}PDGFRβ.

(C) Cortactin and Src were immunoprecipitated from cell lysates of HMLE-Twist1 cells treated with 8 μg/ml PDGFRα blocking antibody (mAb) or vehicle control (ctrl) or HMLE-Twist1 cells expressing indicated shRNAs (control, shC; shPDGFRα, shα1 and shα3) and probed for total cortactin and phosphotyrosine or total Src and pTyr^{419}Src, respectively. Input lysates were probed for β-actin, cortactin, and total Src.

(D) HMLE-Twist1 cells were seeded on 0.2% gelatin and treated for 24 hr with 8 μg/ml PDGFRα blocking antibody (αPDGFRα) or vehicle control and stained for phosphotyrosine (green), F-actin (red), and nuclei (blue). Scale bars are 1 μm for insets, 5 μm for full images.

(E) Quantification of cells with invadopodia. N = 150 cells/sample. *$p < 0.02$.

(F) Quantification of FITC-gelatin degradation. N = 150 cells/sample. *$p < 0.02$.

Error bars are SEM.

See also Figure S4.

against PDGFRα and examined invadopodia formation and matrix degradation. This antibody effectively inhibited PDGFRα activation (Figure 4B), Src activation, and tyrosine phosphorylation of cortactin in HMLE-Twist1 cells (Figure 4C). This PDGFRα blocking antibody significantly inhibited invadopodia formation and tyrosine phosphorylation at invadopodia and suppressed the ability of HMLE-Twist1 cells to degrade FITC-gelatin by over 5-fold (Figures 4D–4F). To verify the results observed with the PDGFRα blocking antibody, we also expressed two independent shRNAs against PDGFRα in HMLE-Twist1 cells to stably suppress and inhibit PDGFRα signaling. Both shRNAs potently suppressed PDGFRα expression (Figure 4B), Src activation, and cortactin phosphorylation (Figure 4C), and effectively suppressed the ability of HMLE-Twist1 cells to degrade matrix (Figure 4F). Importantly, expression or secretion of proteases was not affected by PDGFRα knockdown as measured with gelatin zymography (Figure S4B). Together, these data indicate that PDGFRα expression and activation is required for Twist1-induced invadopodia formation and invasion.

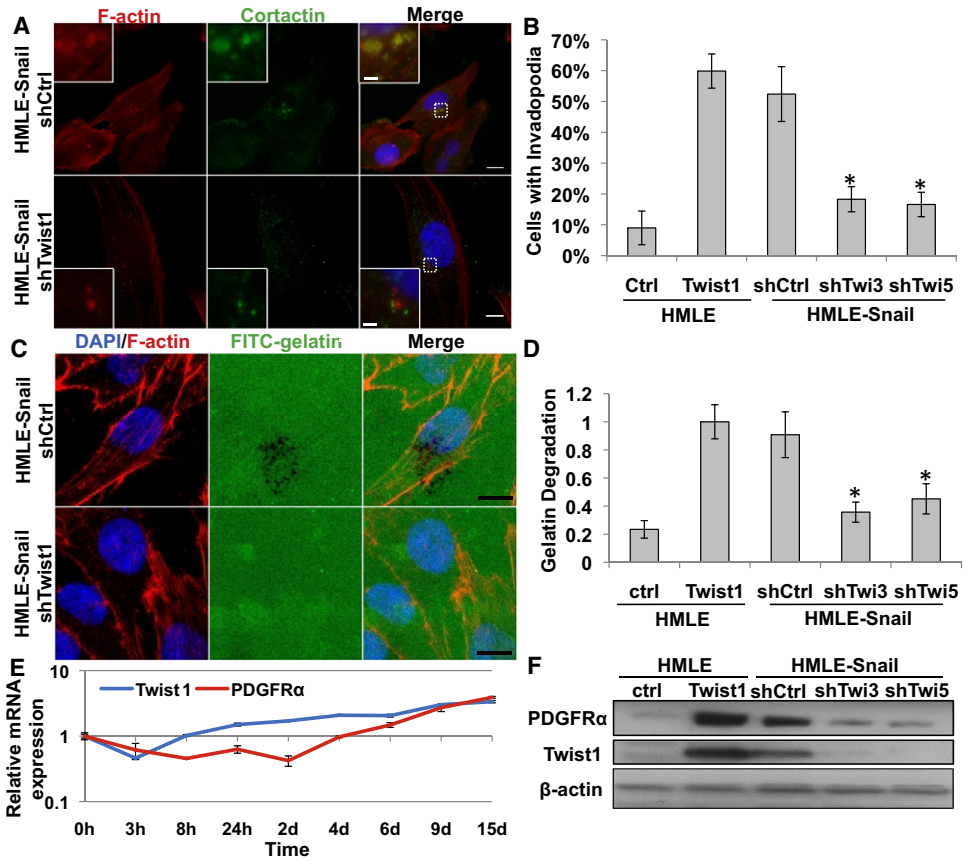

Figure 5. Twist1 Is Required for Snail-Induced Invadopodia Formation

(A) HMLE-Snail cells expressing indicated shRNA were seeded on 0.2% gelatin for 72 hr, and stained for cortactin (green), F-actin (red), and nuclei (blue).

(B) Quantification of cells with invadopodia. N = 150 cells/sample. *$p < 0.02$.

(C) HMLE-Snail cells expressing control or Twist1 shRNA were seeded on FITC-gelatin (green) for 8 hr and stained for F-actin (red) and nuclei (blue).

(D) Quantification of FITC-gelatin degradation. N = 150 cells/sample. *$p < 0.02$.

(E) Real-time PCR analysis of PDGFRα and Twist1 mRNA expression in HMLE-Snail-ER cells treated with 20 nM 4-hydroxy-tamoxifen.

(F) Cell lysates from indicated cells were analyzed by SDS-PAGE and probed for PDGFRα, Twist1, and β-actin.

Error bars are SEM. Scale bars are 1 μm for insets, 5 μm for full images.

See also Figure S5.

We also examined expression of PDGFRα in 168FARN cells expressing control and Twist1 knockdown constructs. PDGFRα was highly expressed in control cells and significantly reduced upon knockdown of Twist1 (Figure S4C). These results provide further evidence that expression of PDGFRα depends on the presence of Twist1 in breast tumor cells.

Twist1 Is a Central Mediator of Invadopodia Formation in Response to EMT-Inducing Signals

Since other inducers of EMT, such as TGFβ and Snail, have also been associated with tumor invasion and metastasis, we sought to understand whether invadopodia formation also occurs in response to other EMT-inducing signals and whether Twist1 mediates invadopodia formation in response to these signals.

To do so, we first tested the ability of Snail, another EMT-inducing transcription factor, to promote invadopodia formation and matrix degradation. As previously reported, Snail overexpression induces EMT similarly to Twist1 in HMLE cells (Mani et al., 2008). HMLE-Snail cells have similar numbers of invadopodia and ECM-degradation activities as HMLE-Twist1 cells (Figures 5A–5D). To determine whether Snail, like Twist1, could induce the expression of PDGFRα to promote invadopodia formation, we examined the expression of PDGFRα mRNA in HMLE cells that express an inducible Snail (Snail-ER) construct. In contrast to the immediate induction of PDGFRα upon Twist1 activation, PDGFRα mRNA only began to increase 6 days after Snail activation, indicating that induction of PDGFRα by Snail is indirect (Figure 5E). Interestingly, endogenous Twist1 mRNA levels increased significantly after 4 days of Snail activation, before PDGFRα mRNA began to increase (Figure 5E). These data suggest that induction of endogenous Twist1 could be responsible for PDGFRα expression and invadopodia formation upon Snail activation.

To assess whether Twist1 mediates the induction of invadopodia and PDGFRα in HMLE-Snail cells, we expressed shRNAs against endogenous Twist1 in HMLE-Snail cells. Indeed, suppression of endogenous Twist1 significantly inhibited expression of PDGFRα in HMLE-Snail cells (Figure 5F).

Significantly, suppression of Twist1 expression inhibited invadopodia formation in HMLE-Snail cells and reduced their ability to degrade matrix (Figures 5A–5D). Importantly, HMLE-Snail cells that express shRNAs against Twist1 presented an EMT phenotype with loss of E-cadherin expression and a mesenchymal morphology (Figures S5A and S5B), indicating that suppression of E-cadherin by Snail and induction of invadopodia by Twist1 are regulated independently. Treating HMLE-Snail cells with the PDGFRα blocking antibody also significantly suppressed the ability of HMLE-Snail cells to degrade FITC-gelatin (Figures S5C and S5D). Together, these results indicate that Twist1 and PDGFRα are responsible for invadopodia formation in response to Snail activation.

To further generalize our finding, we also investigated the role of Twist1 and PDGFRα in regulating invadopodia formation in response to TGFβ. In EpH4 mouse mammary epithelial cells, TGFβ has been shown to collaborate with Ras to promote EMT and activates an autocrine PDGF loop (Jechlinger et al., 2003). When we examined the invadopodia formation and matrix degradation in EpH4-Ras cells treated with TGFβ, we found that TGFβ treatment induced over 5-fold increase of invadopodia formation and matrix degradation in 2D culture (Figures 6A–6C). When these cells grew in 3D culture with TGFβ, invadopodia were visible at the leading edge of cells invading out of the organoids (Figure 6E). Interestingly, both Twist1 and PDGFRα were induced in response to TGFβ treatment (Figure 6D). When endogenous Twist1 induction was inhibited by shRNAs, invadopodia formation and matrix degradation were significantly reduced in 2D and 3D cultures (Figures 6A–6C and 6E). Importantly, knocking down Twist1 abolished induction of PDGFRα in EpH4-Ras cells treated with TGFβ (Figure 6D), but did not prevent induction of EMT morphogenesis and loss of E-cadherin (Figures S6A and S6B), similar to knockdown of Twist1 in HMLE-Snail cells. Furthermore, treating EpH4-Ras cells with the PDGFRα inhibitor ST1571 significantly suppressed their ability to degrade FITC-gelatin in response to TGFβ treatment (Figures S6C and S6D). Importantly, treatment with STI571 did not revert the EMT phenotype (Figure S6E). Together, these results support our conclusion that Twist1 is a central mediator of invadopodia formation and matrix degradation via induction of PDGFRα in response to EMT-inducing signals.

Twist1-Induced Metastasis Is Mediated by Invadopodia In Vivo and Requires PDGFRα

Twist1 is required for mammary tumor cells to metastasize from the mammary gland to the lung. We then tested whether PDGFRα and invadopodia are required for the ability of Twist1 to promote tumor metastasis in vivo. To do so, we generated HMLE-Twist1 cells that were transformed with oncogenic Ras (HMLER-Twist1) and expressed shRNAs against either PDGFRα or a control shRNA. These cells also expressed GFP to allow identification of tumor cells in mice. Individual cell lines were injected subcutaneously into nude mice. Suppression of PDGFRα did not affect cell proliferation in culture or tumor growth rate in vivo (Figures S7A and S7B). Six weeks after tumor implantation, we sacrificed the mice and examined primary tumors for histology and invadopodia. Since HMLER-Twist1 tumors expressing large T antigen, we used an antibody against large T antigen to stain implanted tumor cells. Interestingly, HMLER-Twist1 tumor cells invaded into surrounding stroma and adjacent adipose tissue, while PDGFRa knockdown inhibited local invasion and tumor cells remained encapsulated (Figure 7A). Staining for invadopodia using cortactin and Tks5 in sections of primary tumor tissue revealed that HMLER-Twist1 tumor cells contained abundant invadopodia, while knocking down PDGFRα significantly reduced their occurrence (Figures 7B and 7C). To test whether PDGFRα is required for distant metastasis, examination of lung lobes and sections revealed clusters of HMLER-Twist1 shControl cells throughout the lungs (Figure 7E; Figure S7E). Significantly, suppression of PDGFRα expression significantly reduced the number of disseminated tumor cells in the lung (Figure 7D). These results strongly indicate that induction of PDGFRα is required for the ability of Twist1 to form invadopodia and promote tumor metastasis without affecting primary tumor growth in vivo.

To demonstrate that invadopodia are required for the ability of Twist1 to metastasize in vivo, we expressed shRNAs against Tks5 to inhibit invadopodia formation in HMLER-Twist1 cells. Knockdown of Tks5 did not affect cell growth rate in vitro (Figure S7C), which is consistent with a previous study (Blouw et al., 2008).''. These cells were implanted subcutaneously into nude mice to follow primary tumor growth and lung metastasis. Consistent with the results from the PDGFRα knockdown experiments, Tks5 knockdown inhibited local tumor invasion and significantly reduced the numbers of tumor cells that disseminated into the lung, while primary tumor growth was not affected (Figures 7A, 7D, and 7E; Figure S7D). Together, these data demonstrate that induction of invadopodia formation via PDGFRα activation is essential for the ability of Twist1 to promote tumor metastasis in vivo.

PDGFRα Is a Direct Transcription Target of Twist1 and Expression of Twist1 and PDGFRα Are Tightly Linked in Human Breast Tumors

Given the immediate induction of PDGFRα by Twist1 and their tight association in various tumor cells, we set out to determine whether PDGFRα is a direct transcriptional target of Twist1. We examined the human PDGFRα promoter for potential Twist1-binding E-box sequences (CANNTG). We designed three sets of primers on the putative promoter: primer sets 1 and 2 target the identified E-box, and primer set 3 targets an adjacent region lacking the putative E-box (Figure 8A). By chromatin immunoprecipitation, we found that Twist1 directly bound to the E-box on the putative PDGFRα promoter (Figure 8B). Twist1 was able to activate the isolated human PDGFRa promoter in an E-box-dependent fashion in a luciferase reporter assay (Figures S8A and S8B). Furthermore, this consensus E-box sequence is highly conserved between all mammalian species examined and chickens (Figure 8C), indicating that induction of PDGFRα by Twist1 is direct and evolutionally conserved.

To more directly probe the in vivo association between Twist1 and PDGFRα in human breast tumor samples, we analyzed four published large human breast tumor gene expression data sets summarizing 860 primary breast cancers (Pawitan et al., 2005; Sotiriou et al., 2006; Wang et al., 2005; Miller et al., 2005). In each data set, we calculated the rank-based Spearman correlation coefficient between Twist1 and all 22282 genes on the array, including PDGFRa. PDGFRα was consistently among the top

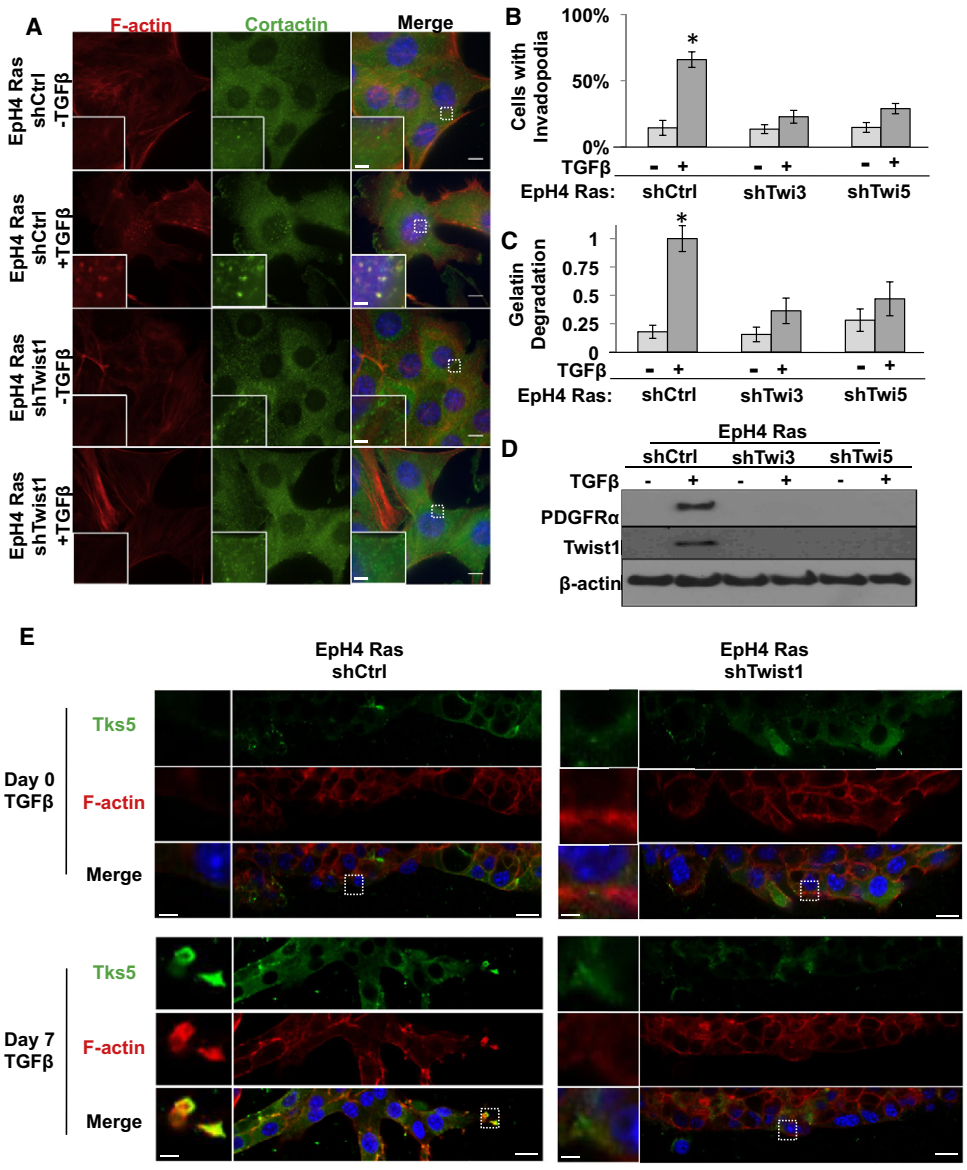

Figure 6. Twist1 Is Required for TGFβ-Induced Invadopodia Formation in Eph4Ras Cells

(A) Eph4Ras cells expressing control or Twist1 shRNAs were seeded on 0.2% gelatin for 72 hr before and after treatment with 5 ng/ml TGFβ1 for 7 days and stained for cortactin (green), F-actin (red), and nuclei (blue).

(B) Quantification of cells with invadopodia before and after 7 days of 5 ng/ml TGFβ1 treatment for EpH4Ras cells expressing indicated shRNAs. N = 150 cells/sample. *$p < 0.02$.

(C) Quantification of FITC-gelatin degradation for cells expressing indicated shRNA before and after 7 days of 5 ng/ml TGFβ1 treatment. N = 150 cells/sample. *$p < 0.02$.

(D) Cell lysates from indicated cells before and after treatment with 5 ng/ml TGFβ1 for 7 days were analyzed by SDS-PAGE and probed for PDGFRα, Twist1, and β-actin.

(E) Indicated cells were embedded in 1:1 mixture of Matrigel and collagen, allowed to form 3D structures, and processed for IF before and after 7 days of induction with 7 ng/ml TGFβ1. Cells were stained for Tks5 (green) and F-actin (red).

Error bars are SEM. Scale bars are 1 μm for insets, 5 μm for full images.

See also Figure S6.

ranked genes associated with Twist1 (4th, 17th, 47th, and 54th out of 22,282 genes) in all four breast cancer data sets (Figure 8D; Figure S8D). Expression of Twist1 and PDGFRα were positively correlated with correlation coefficients ranging from 0.56 to 0.70 (Figure 8D; Figure S8C). Furthermore, in all four data sets, PDGF ligand expression correlated with PDGFRα and Twist1 expression in over 95% of tumor samples (Table S1), indicating that PDGFRα could be active in these samples. To further access

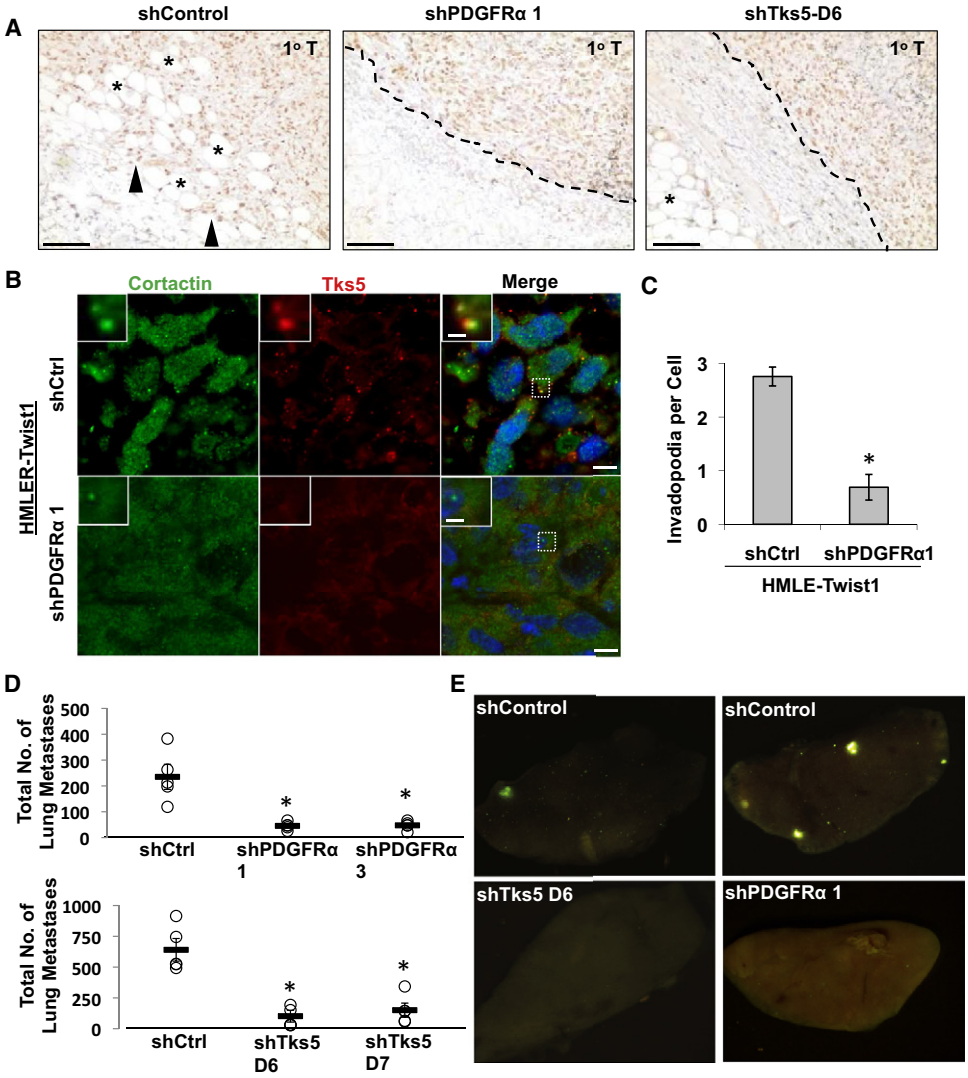

Figure 7. Twist1-Induced Metastasis Is Mediated by Invadopodia In Vivo and Requires PDGFRα

(A) Representative images of primary tumor paraffin tissue sections stained with SV40 Large-T antigen IHC and counterstained with hematoxylin. Tumor margin is indicated with dashed line when apparent. Closed triangles indicate invasive, Large-T positive tumor cells. Asterisks indicate adjacent adipose tissue. Scale bars are 100 μm.

(B) Images of sections of primary tumors stained with cortactin (green), Tks5 (red), and DAPI (blue). Scale bars are 1 μm for insets, 5 μm for full images.

(C) Quantification of number of invadopodia (cortactin/Tks5 colocalization) per cell. N = 150 cells/sample. *$p < 0.02$.

(D) Quantification of total number of GFP positive tumor cells (HMLER-Twist1 cells expressing indicated shRNAs) in individual lungs. N = 5 mice per group.

(E) Representative images of lungs from mice injected with HMLER-Twist1 cells expressing indicated shRNAs show a decrease in dissemination of GFP positive tumor cells (green) to the lungs upon knockdown of PDGFRα or Tks5.

Error bars are SEM.

See also Figure S7.

whether coexpression of Twist1 and PDGFRα could affect survival in breast tumor patients, we stained Twist1 and PDGFRα in a set of human invasive breast tumor tissue array samples and found that coexpression of Twist1 and PDGFRα was negatively associated with long-term survival (Figures 8E and 8F). Together, these data provide further support for a direct and functional association between Twist1 and PDGFRα in human breast cancers and suggest that regulation of invadopodia by Twist1 and PDGFRα contributes to human breast cancer progression.

DISCUSSION

Our study has identified a unique function of the Twist1 transcription factor in promoting invadopodia formation and matrix degradation during tumor metastasis. We demonstrate that transcriptional induction of PDGFRα and activation of Src by Twist1 are essential for invadopodia formation and matrix degradation. Induction of PDGFRα and invadopodia formation is also essential for the ability of Twist1 to promote metastasis in vivo. Twist1

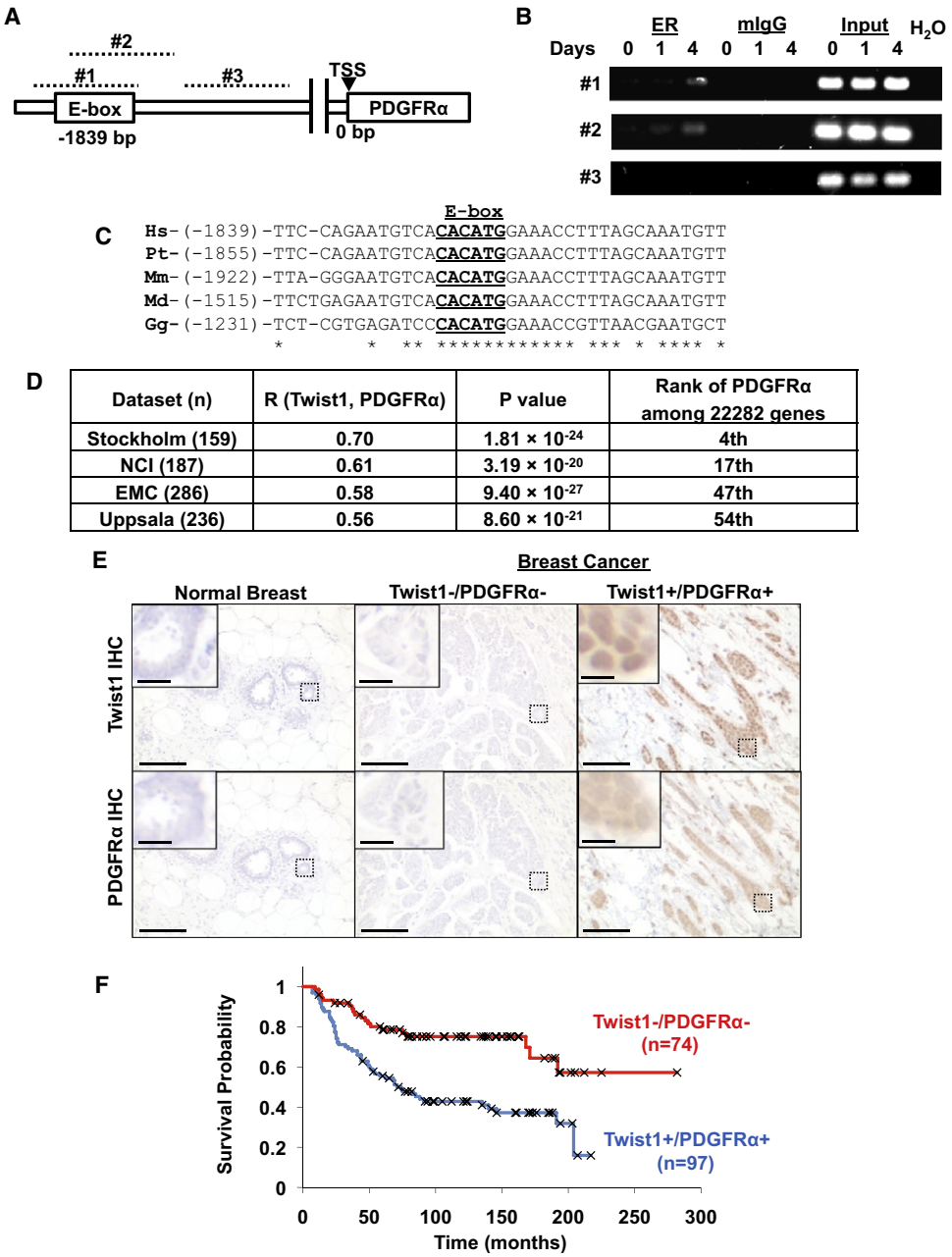

Figure 8. PDGFRα Is a Direct Target of Twist1 and Expression of Twist1 and PDGFRα Is Negatively Correlated with survival

(A) Schematic of the human PDGFRα gene promoter region with conserved E-box element 1839 bp upstream of transcriptional start site (TSS), and regions targeted by three primer pairs (#1-3, dashed lines). Primer pairs #1 and #2 target the putative E-box while primer pair #3 targets a downstream region lacking a conserved E-box.

(B) HMLE-Twist1-ER cells were treated with 20 nM 4-hydroxytamoxifen for 0, 1, or 4 hr. Chromatin was immunoprecipitated using estrogen receptor antibody and PCR was performed on the ChIP product using three primer pairs.

(C) Alignment of conserved E-box (underlined) in PDGFRα promoter. Number in parenthesis indicates distance upstream of transcription start site. Hs = *Homo sapiens*, Pt = *Pan troglodytes*, Mm = *Mus musculus*, Md = *Monodelphus domesticus*, Gg = *Gallus gallus*.

(D) Correlation of Twist1 and PDGFRα in four human breast cancer expression array data sets. R is the correlation coefficient.

(E) Representative images of normal human breast tissue or human breast cancer samples stained for Twist1 and PDGFRα. Scale bar is 5 μm for inset, 100 μm for full images.

(F) Kaplan-Meier survival curve for samples classified as high PDGFRα/high Twist1 expression and low PDGRα/low Twist1 by IHC analysis. Censored data are indicated with X.

See also Figure S8 and Table S1.

and PDGFRα are central mediators of invadopodia in response to several EMT-inducing signals. Finally, we provide evidence for a tight association between Twist1 and PDGFRα in human breast tumor samples.

Induction of Invadopodia by Twist1 Plays a Key Role in Extracellular Matrix Degradation and Metastasis

ECM degradation is considered a key step promoting tumor invasion and metastasis. Extensive studies have largely focused on secreted MMPs as key proteases in tumor invasion. More recent studies suggest a role for invadopodia and their associated proteases in localized matrix degradation during cell invasion. Conceptually, invadopodia provide an elegant solution to restrict protease activity to areas of the cell in direct contact with ECM, thus precisely controlling cell invasion in vivo. In this study, we show that Twist1, a key transcription factor in tumor metastasis, is both necessary and sufficient to promote invadopodia formation. Importantly, invadopodia formation is required for the ability of Twist1 to promote tumor metastasis in vivo. Together, these results demonstrate an essential role for invadopodia in tumor invasion and metastasis in vivo.

How invadopodia formation is regulated at the molecular level is still not well understood. Our current study indicates that Twist1 directly induces the expression and activation of PDGFRα, thus promoting Src kinase activation and invadopodia formation. Although we did not detect induction of several important invadopodia proteins, including cortactin, Tks4, Tks5, and MT1-MMP, by Twist1 (data not shown), we are actively exploring additional mechanisms by which Twist1 regulates invadopodia.

Another question arising from our study is whether invadopodia function is required for the EMT process. Epithelial cells sit on top of a layer of basement membrane. For the EMT program to occur in vivo, these cells must breach the underlying basement membrane to dissociate (Nakaya et al., 2008). Little is known about the functional relationship between basement membrane integrity and the EMT program. In HMLE-Snail cells and EpH4-Ras cells treated with TGFβ, knockdown of Twist1 inhibited invadopodia formation, while these cells underwent the morphological changes associated with EMT and lost E-cadherin expression. Additionally, knockdown of Tks5, a required component of invadopodia, did not revert the EMT phenotype in HMLE-Twist1 cells. These results indicate that invadopodia function is not essential for EMT to occur in 2D cultures. However, it is plausible that the EMT program requires activation of Twist1 and invadopodia formation to allow degradation of the basement membrane in vivo. Studies in vivo or in 3D cultures with intact basement membrane are required to fully answer this question.

Twist1 and Snail Have Distinct Cellular Functions and Transcriptional Targets

The EMT program is considered a key event promoting carcinoma cell dissociation, invasion, and metastasis. Several transcription factors, including Snail, Slug, ZEB1, ZEB2, and Twist1, promote EMT in epithelial cells (Peinado et al., 2007). During mesoderm formation and neural crest development, these transcription factors are activated to allow the dissociation and migration of epithelial cells. A major unsolved question is to determine the distinct cellular functions and molecular targets of individual EMT-inducing transcription factors. Extensive studies in recent years have demonstrated that Snail (Batlle et al., 2000; Cano et al., 2000), Slug (Hajra et al., 2002), and ZEB2 (Comijn et al., 2001), all zinc-finger-containing transcriptional repressors, directly bind to the E-boxes on the E-cadherin promoter and suppress its transcription. In this study, we identified a unique function of Twist1 in promoting matrix degradation via invadopodia. We show that Twist1 functions as a transcriptional activator to directly induce the expression of PDGFRα, in contrast to the EMT-inducing Zn-finger transcription factors.

Vertebrate Twist1 lacks a transcription activation domain and requires dimerization with other bHLH transcription factors to activate transcription. Previous studies have shown Twist1 heterodimers with MyoD function as transcriptional repressors (Hamamori et al., 1997). In contrast, heterodimerization with E12 enables Twist1 to activate FGF2 transcription (Laursen et al., 2007). Here, we demonstrate that Twist1 functions as a transcriptional activator to directly induce the transcription of PDGFRα. Twist1 might function as an activator or repressor of transcription based on dimerization partners under different physiological and cellular environments. The factors that heterodimerize with Twist1 to activate PDGFRα transcription remain unknown, although the E12/E47 proteins could perform this function.

The Pathway Linking Twist1, PDGFR, and Invadopodia Is Likely to Play a Conserved Role in Matrix Degradation during Both Tumor Metastasis and Embryonic Morphogenesis

Twist1 has been associated with increased metastasis in both experimental tumor metastasis models and in many types of human cancers. Interestingly, PDGFRα overexpression and activation have also been observed in aggressive human breast tumors (Seymour and Bezwoda, 1994; Jechlinger et al., 2006). Activation of PDGFRs was first observed in TGFβ-induced EMT and shown to be involved in cell survival during EMT and experimental metastasis in mice (Jechlinger et al., 2006). Here, we demonstrated a role of PDGFRα in invadopodia formation and matrix degradation during tumor metastasis. Interestingly, suppression of PDGFRα had no significant effects on cell proliferation or survival in vitro and in vivo. These results could be due to the greater specificity of shRNAs compared with chemical inhibition as well as differences in cellular and signaling contexts. Indeed, we found that STI571 (Gleevec), a c-ABL and c-Kit inhibitor that also inhibits PDGFR at a higher concentration, suppressed Twist1-induced invadopodia formation and matrix degradation. However, long-term (4 days) treatment with STI571 resulted in cell toxicity in HMLE-Twist1 cells (data not shown).

Our analyses identified Twist1 as a transcription inducer of PDGFRα and demonstrate a tight correlation between the expression level of Twist1 and PDGFRα in four large human breast tumor gene expression studies. Interestingly, PDGF ligand was also present in over 95% of tumor samples that expressed Twist1 and PDGFRα, indicating PDGFRα is activated in these tumors. Although these two genes alone are not sufficient to predict survival with statistical significance in these studies, these data, together with our metastasis data in mice and human breast cancer tissue array data, strongly suggest

their involvement in breast cancer progression. Twist1, as a transcription factor, is difficult to target therapeutically. As a downstream target of Twist1 with roles in tumor invasion, PDGFRα might be a potentially valuable target for future therapeutics against metastasis.

Although our study focuses on the role of Twist1-induced invadopodia in metastasis, it also has important implications in development. Twist1 null mice and PDGFRα null mice both show defects in cranial neural crest development (Chen and Behringer, 1995, Sun et al., 2000). In addition, the expression pattern of Twist1 and PDGFRα are similar along the developing neural crest and craniofacial region in developing mouse embryos (Gitelman, 1997, Takakura et al., 1997). Our identification of PDGFRα as a highly conserved transcriptional target of Twist1 suggests that the pathway linking Twist1, PDGFRα, and invadopodia might play a key role in regulating neural crest development. Reactivation of this developmental machinery in tumor metastasis is another example of an important developmental pathway regulating tumor progression.

EXPERIMENTAL PROCEDURES

Cell Lines

67NR, 168FARN, 4T1 cells and the human mammary epithelial cell lines HMLE and HMLER were cultured as described (Yang et al., 2004). EpH4Ras cells were passaged in mammary epithelial growth media (MEGM) mixed 1:1 with DMEM/F12 supplemented with human EGF, insulin, and hydrocortisone.

Viral Production and Infection

Stable cell lines were created via infection of target cells using either lentiviruses or Moloney viruses. 293T cells were seeded at 1×10^6 cells per 6 cm dish in DMEM/10%FBS. After 18 hr, cells were transfected as follows: 6 μl TransIT-LT1 (Mirus Bio) was added to 150 μl DMEM and incubated 20 min. One microgram of viral vector along with 0.9 μg of the appropriate gag/pol expression vector (pUMCV3 for pBabe or pWZL or pCMVΔ8.2R for lentiviral vectors) and 0.1 μg VSVG expression vector were then added to the DMEM/LT-1 mixture. The mixture was incubated 30 min and then added to 293T cells overnight. Next day fresh media were added to the transfected 293T cells. Viral supernatant was harvested at 48 and 72 hr posttransfection, filtered, and added to the recipient cell lines with 6 μg/ml protamine sulfate for 4 hr infection. HMLE and EpH4Ras cells were then selected with 2 μg/ml puromycin, or 10 μg/ml blasticidin.

Plasmids

The Twist1 and Snail cDNAs and the Twist1-ER and Snail-ER in the pWZL-Blast vector were described in Mani et al., 2009. The three shRNA lentiviral constructs against Twist1 in the pSP108 vector were described in Yang et al. (2004). The shRNA lentiviral constructs against Tks5 in the pLKO vector were provided by Dr. Sara Courtneidge. The shRNAmir lentiviral constructs against PDGFRα in the pGIPZ vector were purchased from Open Biosystems. The oncogenic Ras (V12) was cloned into the pRRL lentiviral vector.

Real-Time PCR

Total RNAs were extracted from cells at 80%–90% confluency using RNeasy Mini Kit coupled with DNase treatment (QIAGEN) and reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Resulting cDNAs were analyzed in triplicates using SYBR-Green Master PCR mix (Applied Biosystems). Relative mRNA concentrations were determined by $2^{-(Ct-Cc)}$ where Ct and Cc are the mean threshold cycle differences after normalizing to GAPDH values. Primers used for PCR are listed in Supplemental Experimental Procedures.

Immunoprecipitation

Cells at 80%–90% confluence were washed with PBS containing 100 μM Na_3VO_4 and lysed in lysis buffer (50 mM Tris-HCL [pH 7.5], 150 mM NaCl, 1% Triton X-100, 10 mM NaF, 100 μM Na_3VO_4, 2 mM DTT) containing 1:50 dilution Protease Inhibitor Cocktail Set III (Calbiochem). For immunoprecipitations, lysates were incubated with antibodies overnight at 4°C. Fifty microliters Protein G-Sepharose 4B conjugated beads (Invitrogen) was added for 12 hr at 4°C. Beads were washed in lysis buffer and in PBS containing 100 μM Na_3VO_4. Proteins were eluted from beads using SDS sample buffer and analyzed on 4%–12% precast SDS gels (PAGEgel).

In Situ Zymography

This protocol is adapted from Artym et al. (2009). In brief, 12 mm coverslips were incubated in 20% nitric acid for 2 hr and washed in H_2O for 4 hr. Coverslips were incubated with 50 μg/ml poly-L-lysine/PBS for 15 min followed by PBS washes before 0.15% gluteraldehyde/PBS was added for 10 min, followed by PBS washes. Coverslips were inverted onto 20 μl droplets of 1:9 0.1% fluorescein isothiocyante (FITC)-gelatin (Invitrogen): 0.2% porcine gelatin for 10 min. Coverslips were washed in PBS and then incubated 15 min in 5 mg/ml $NaBH_4$. Coverslips were rinsed in PBS and incubated at 37° in 10% calf serum/DMEM for 2 hr. Twenty thousand cells were seeded on each coverslip, incubated for 8 hr, and processed for immunofluorescence. Each experiment was performed in triplicate. Images were taken at ten fields per sample for a total of approximately 150 cells per sample. Gelatin degradation was quantified using ImageJ software. To measure the percentage of degraded area in each field, identical signal threshold for the FITC-gelatin fluorescence are set for all images in an experiment and the degraded area with FITC signal below the set threshold was measured by ImageJ. The resulting percentage of degradation area was further normalized to total cell number (counted by DAPI staining for nuclei) in each field. The final gel degradation index is the average percentage degradation per cell obtained from all ten fields. Each experiment was repeated at least three times.

Immunofluorescence

Matrix substrates were prepared using 0.2% porcine gelatin as for in situ zymography. Cells were fixed at 37°C in 4% paraformaldehyde (PFA)/PBS with 50 μM $CaCl_2$ for 15 min, permeabilized with 0.1% Triton X-100/PBS for 10 min, and blocked with 5% goat serum. Samples were incubated with primary antibodies overnight at 4°C and with secondary antibodies and/or phalloidin for 2 hr. After washing, coverslips were mounted with VECTASHIELD (Vector Laboratories). All antibodies used and their dilutions are listed in Supplemental Experimental Procedures.

Chromatin Immunoprecipitation

Cells at 80% confluence were crosslinked with 4% PFA, lysed, and sonicated. Nuclear lysates were incubated with Protein G Dynabeads (Invitrogen) preconjugated with antiestrogen receptor antibody overnight. DNA was reverse crosslinked and purified by phenol-chloroform and ethanol precipitation.

Subcutaneous Tumor Implantation and Metastasis Assay

All animal care and experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, San Diego. Cells (1.5 million) resuspended in 50% Matrigel were injected into the left and right flanks of Nude mice and allowed to grow to about 2 cm in diameter before mice were sacrificed. Primary tumor size was measured every 5 days. Lungs were harvested and imaged for GFP positive tumor cells. Tissues were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and imaged to identify GFP positive tumor cells.

Three-Dimensional Cell Culture

Equal volumes of neutralized collagen I and Matrigel were mixed on ice and 20 μl added to the bottom of each well of an eight chamber coverglass slide. Cells of interest were mixed with the Matrigel:collagen mix to give a final concentration of 200,000 cells per ml and 100 cells μl of the cell:matrix mixture added to each well. Media was changed every other day until establishment of spherical colonies. TGFβ1 was added at 5 ng/ml every other day for up to 2 weeks. Cells were fixed with 4% PFA and processed as described above for immunofluorescence.

Immunohistochemistry

Paraffin sections of human or mouse samples were rehydrated through xylene and graded alcohols. Antigen retrieval was accomplished using a pressure cooker in 10 mM sodium citrate 0.05% Tween. Samples were incubated with 3% H_2O_2 for 30 min followed by 5 hr blocking in 20% goat serum in PBS. Endogenous biotin and avidin were blocked using a Vector Avidin/Biotin blocking kit. Primary antibodies were incubated overnight at 4°C in 20% goat serum. Biotinylated secondary antibody and Vectorstain ABC kit were used as indicated by manufacturer. Samples were developed with diaminobenzidine (DAB) and samples counterstained with hematoxylin and mounted with Permount.

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures, one table, and Supplemental Experimental Procedures and can be found online at doi:10.1016/j.ccr.2011.01.036.

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Cancer Cell, *19*

Supporting Information

Twist1-Induced Invadopodia Formation

Promotes Tumor Metastasis

Mark A. Eckert, Thinzar M. Lwin, Andrew T. Chang, Jihoon Kim, Etienne Danis, Lucila Ohno-Machado, and Jing Yang

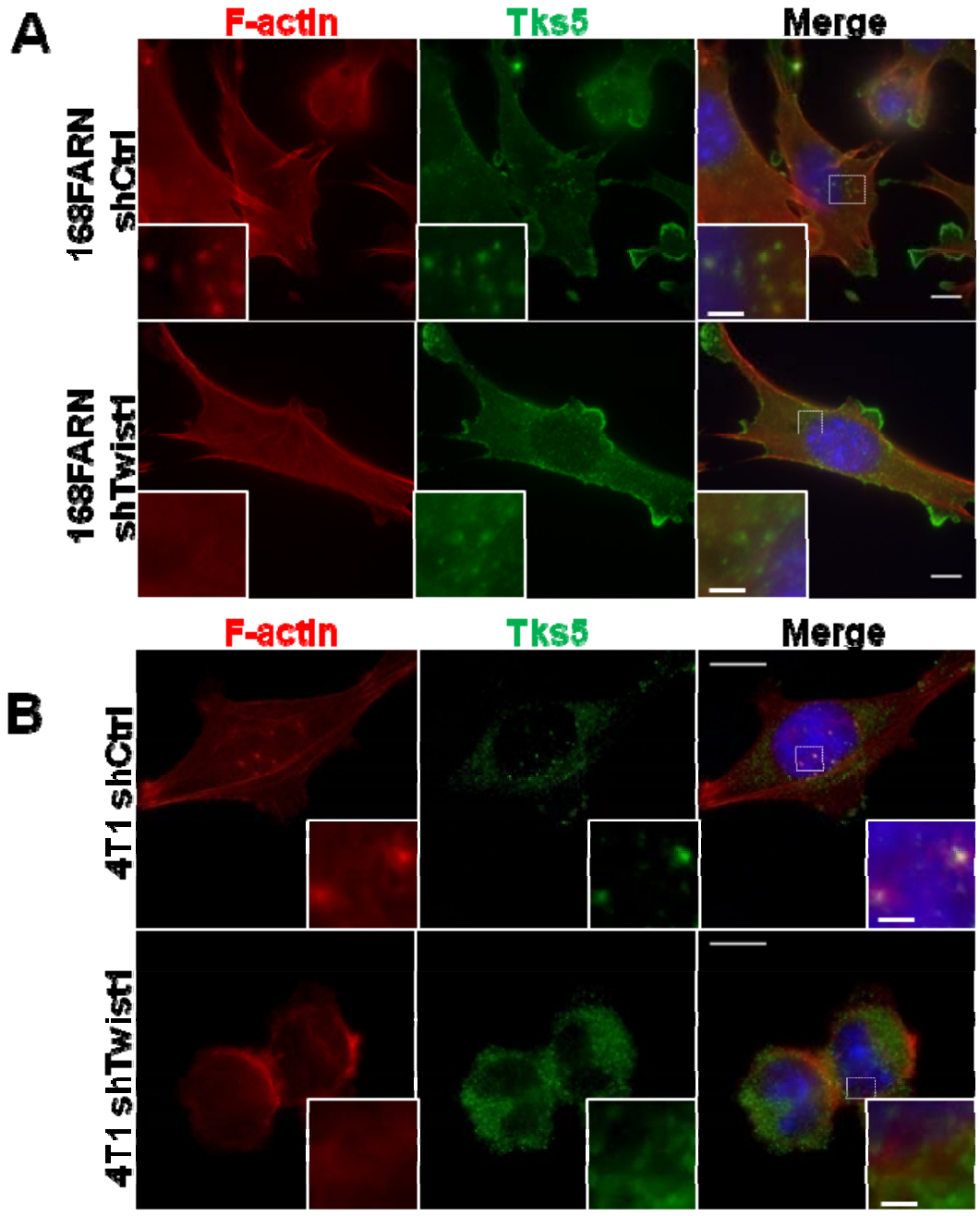

A
F-actin
Tks5
Merge
168FARN shCtrl
168FARN shTwist1
B
F-actin
Tks5
Merge
4T1 shCtrl
4T1 shTwist1

Figure S1: Tks5 localization to invadopodia is abolished upon knockdown of Twist1 in 168FARN and 4T1 cells, related to Figure 1.

A. 168FARN cells expressing indicated shRNA were seeded on 0.2% gelatin for 72 hours and stained for F-actin (red) with phalloidin, Tks5 (green), and nuclei with DAPI (blue). Invadopodia can be visualized as colocalization of phalloidin with Tks5.
B. 4T1 cells expressing indicated shRNA were seeded on 0.2% gelatin for 72 hours and stained for F-actin (red), Tks5 (green), and nuclei (blue). Scale bar is 1 μm for insets, 5 μm for full images.

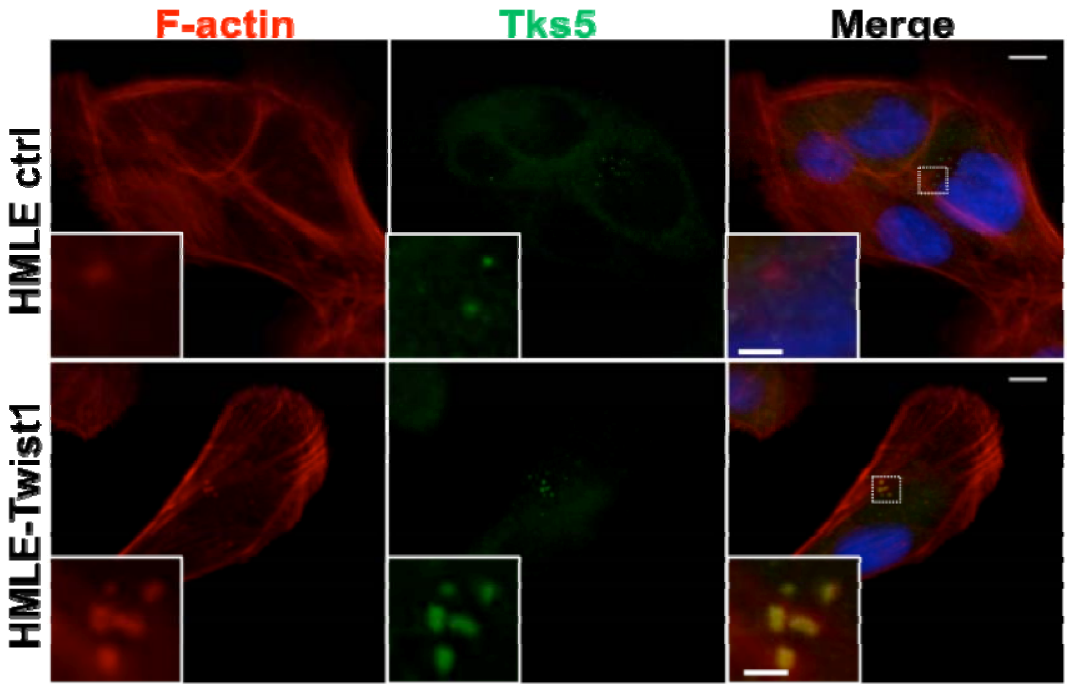


Figure S2: Twist1-induced invadopodia contains Tks5, related to Figure 2.
HMLE cells expressing control or Twist1 vector were plated on 0.2% gelatin for 72 hours and stained for F-actin with phalloidin (red), Tks5 (green), and nuclei with DAPI (blue). Tks5 colocalizes with F-actin at invadopodia upon Twist1 overexpression. Scale bar is 1 μm for insets, 5 μm for full images.

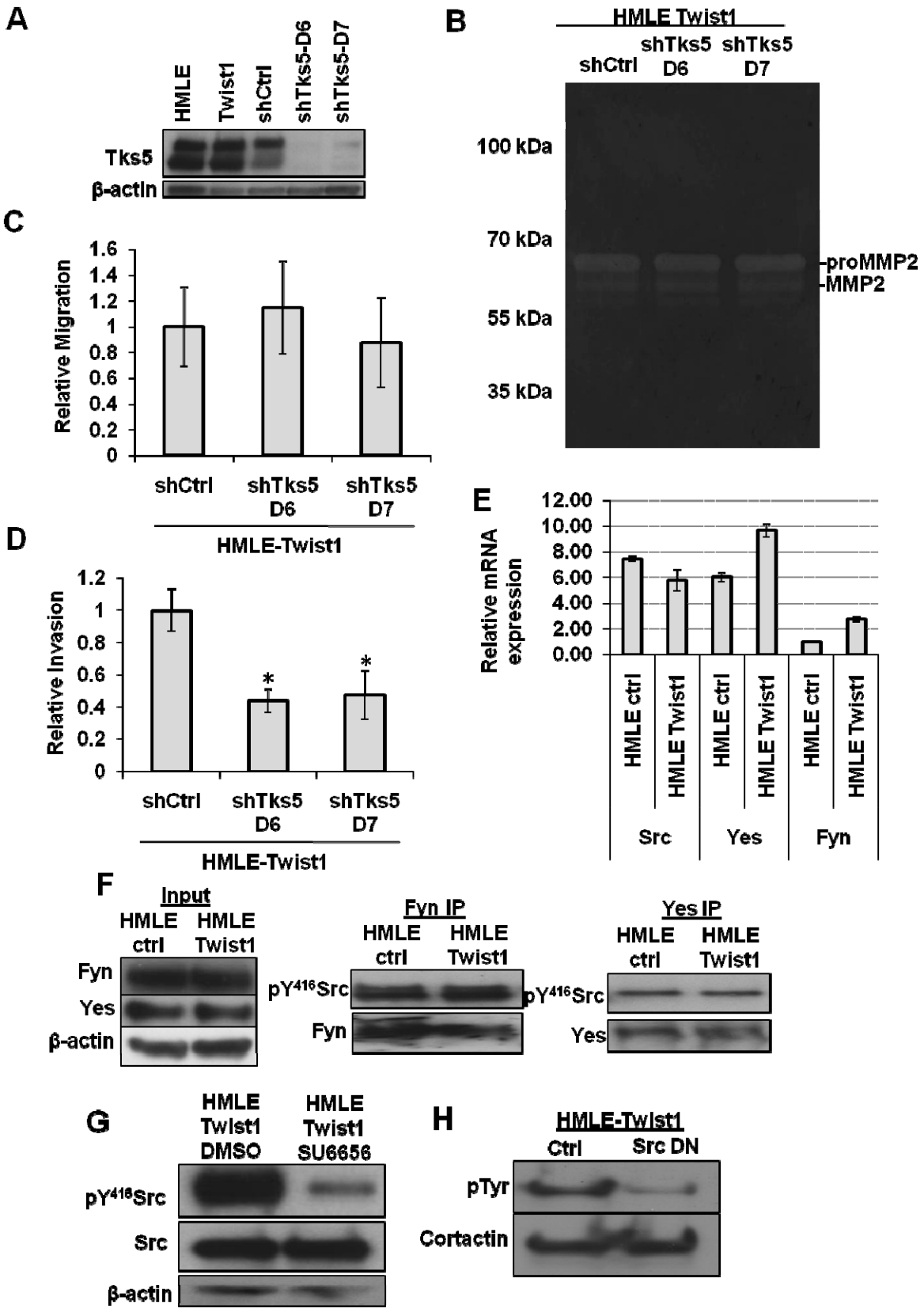
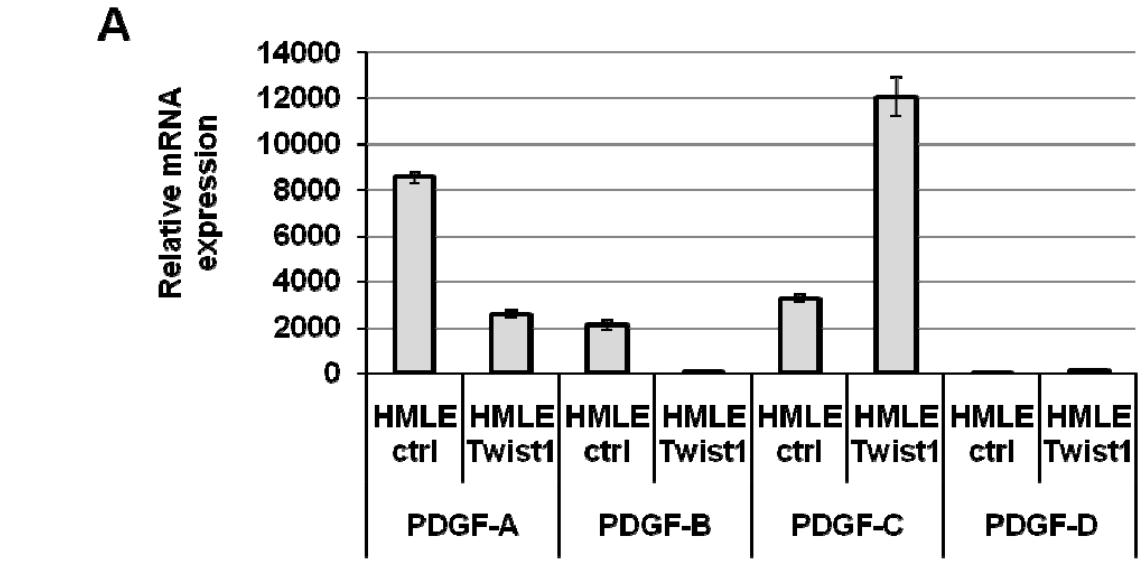
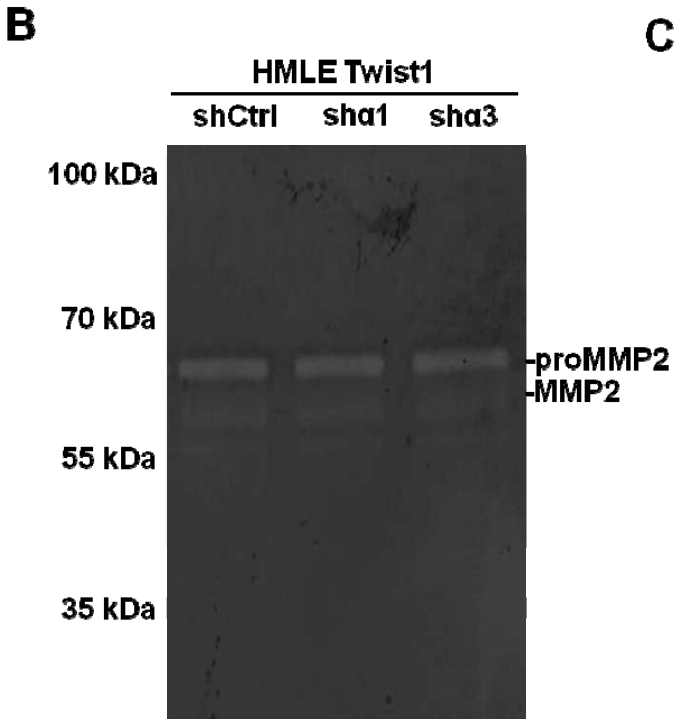

A
HMLE
Twist1
shCtrl
shTks5-D6
shTks5-D7
Tks5
β-actin
B
HMLE Twist1
shCtrl
shTks5 D6
shTks5 D7
100 kDa
70 kDa
55 kDa
35 kDa
proMMP2
MMP2
C
Relative Migration
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
shCtrl
shTks5 D6
shTks5 D7
HMLE-Twist1
D
Relative Invasion
1.2
1
0.8
0.6
0.4
0.2
0
*
*
shCtrl
shTks5 D6
shTks5 D7
HMLE-Twist1
E
Relative mRNA expression
12.00
10.00
8.00
6.00
4.00
2.00
0.00
HMLE ctrl
HMLE Twist1
HMLE ctrl
HMLE Twist1
HMLE ctrl
HMLE Twist1
Src
Yes
Fyn
F
Input
HMLE ctrl
HMLE Twist1
Fyn
Yes
β-actin
Fyn IP
HMLE ctrl
HMLE Twist1
pY416Src
Fyn
Yes IP
HMLE ctrl
HMLE Twist1
pY416Src
Yes
G
HMLE Twist1 DMSO
HMLE Twist1 SU6656
pY416Src
Src
β-actin
H
HMLE-Twist1
Ctrl
Src DN
pTyr
Cortactin

Figure S3: Twist1-induced invasion is mediated by invadopodia and requires Src activation, related to Figure 3.

A. Cell lysates from HMLE-Twist1 cells expressing control or Tks5 shRNA were analyzed by SDS-PAGE and probed for Tks5 and β-actin. Both shRNAs against Tks5 were effective at knocking down Tks5 at the protein level.
B. Conditioned media from HMLE-Twist1 cells expressing control or Tks5 shRNA were analyzed with gelatin zymography. No differences in protease activity can be observed between control and shTks5 knockdown cells. Bands correlating to expression of pro-MMP2 and cleaved, active MMP2 are indicated.
C. 40,000 HMLE-Twist1 cells expressing control or Tks5 shRNAs were plated on Transwell inserts. Cells that migrated through the insert were stained with crystal violet and quantified by releasing dye with 10% acetic acid and measuring absorbency at 520 nm. No significant differences in migration were observed. Error bars are SEM.
D. 40,000 HMLE-Twist1 cells expressing control or Tks5 shRNAs were plated on Transwell inserts coated with a thin layer of Matrigel to assay invasion. Invasion was quantified identically as in C. Knockdown of Tks5 caused a significant reduction in invasion by HMLE-Twist1 cells. Error bars are SEM. * $p<0.02$.
E. mRNA collected from HMLE and HMLE-Twist1 cells was reverse-transcribed and analyzed with real-time PCR for expression levels of three Src family kinases: Src, Fyn, and Yes. Values were normalized against GAPDH values. Error bars are SEM.
F. The Src family kinases Yes and Fyn were immunoprecipitated from lysates of HMLE control and HMLE-Twist1 cells and analyzed by SDS-PAGE. Blots were probed with Yes and Fyn antibodies as well as a $pTyr^{416}$Src antibody that recognizes the active (phosphorylated) form of all Src family kinases. No differences in overall protein expression or activation level are observed for Yes or Fyn.
G. HMLE-Twist1 cells were treated for eight hours with 5 μM SU6656 or DMSO control. Cell lysates were harvested and analyzed by SDS-PAGE. Blots were probed for total Src and active $pTyr^{416}$Src. Total Src levels remain unchanged while the proportion of active Src is significantly reduced.
H. Lysates from HMLE-Twist1 cells transfected with either control or dominant-negative Src (DN-Src) constructs were harvested and immunoprecipitated with an antibody against cortactin, analyzed by SDS-PAGE, and probed for cortactin and phosphotyrosine.


A
Relative mRNA expression
14000
12000
10000
8000
6000
4000
2000
0
HMLE ctrl
HMLE Twist1
HMLE ctrl
HMLE Twist1
HMLE ctrl
HMLE Twist1
HMLE ctrl
HMLE Twist1
PDGF-A
PDGF-B
PDGF-C
PDGF-D


B
HMLE Twist1
shCtrl
shα1
shα3
100 kDa
70 kDa
55 kDa
35 kDa
proMMP2
MMP2

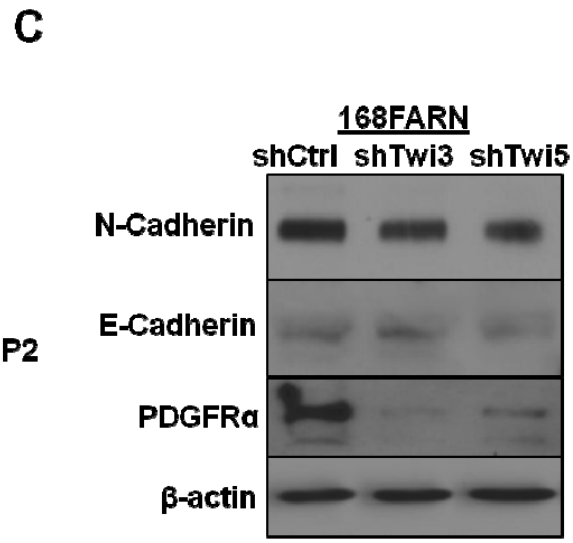

C
168FARN
shCtrl
shTwi3
shTwi5
N-Cadherin
E-Cadherin
PDGFRα
β-actin

Figure S4: PDGFRα is not required for protease secretion and is likely activated by PDGF-C and knockdown of Twist1 does not revert EMT in 168FARN cells related to Figure 4.

A. mRNAs from HMLE control and HMLE-Twist1 cells were analyzed for expression of all PDGF ligands (PDGF-A, PDGF-B, PDGF-C, and PDGF-D) and normalized against GAPDH expression. Error bars are SEM.
B. Conditioned media from HMLE-Twist1 cells expressing control or PDGFRα shRNA were analyzed with gelatin zymography. No difference in protease activity was observed between control and shPDGFRα knockdown cells. Bands representing pro-MMP2 and cleaved, active MMP2 are indicated.
C. Lysates from 168FARN cells expressing indicated shRNA were analyzed by SDS-PAGE and probed for N-cadherin, E-cadherin, PDGFRα, and β-actin.

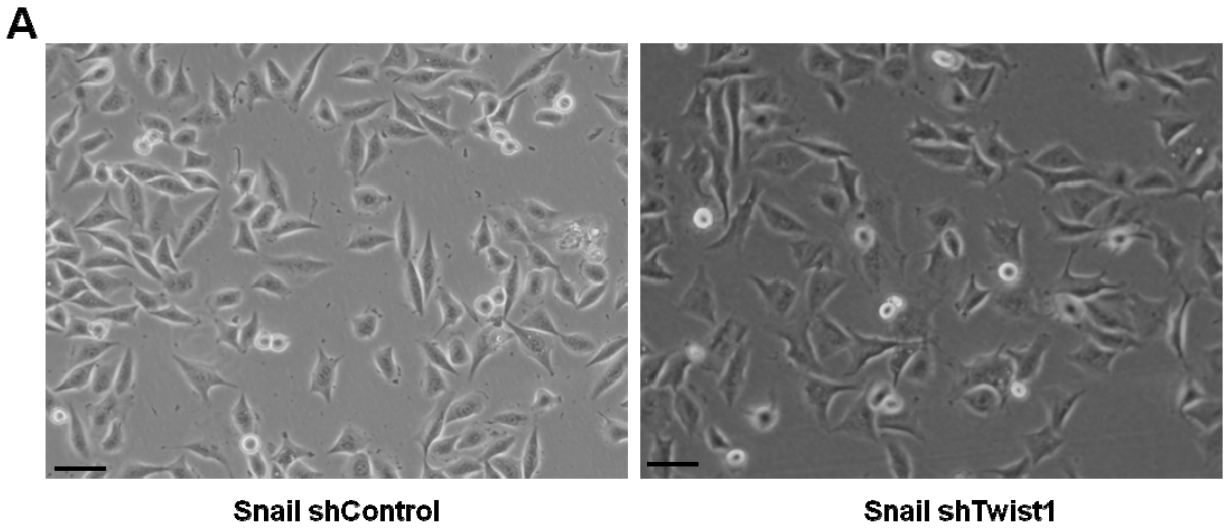

A
Snail shControl
Snail shTwist1
B
HMLE
HMLE-Snail
ctrl
Twist1
shCtrl
shTwi3
shTwi5
E-cadherin
N-cadherin
β-actin
C
HMLE-Snail
Vehicle
αPDGFRα
pY-PDGFRα
PDGFRα
β-actin
D
Gelatin Degradation
1.2
1
0.8
0.6
0.4
0.2
0
*
*
HMLE ctrl
Vehicle
αPDGFRα
HMLE-Snail

Figure S5: Knockdown of Twist1 in HMLE-Snail cells does not revert the EMT phenotype and inhibition of PDGFRα suppresses matrix degradation in HMLE-Snail cells, related to Figure 5.

A. Bright-field images of HMLE-Snail cells expressing control or Twist1 shRNA. Both cells have similar mesenchymal morphologies with loss of cell adhesions and cell scattering. Scale bars are 10 μm.
B. Cell lysates from HMLE control, HMLE-Twist1, and HMLE-Snail cells expressing indicated shRNAs were analyzed by SDS-PAGE and probed for markers of mesenchymal cells (N-cadherin) and of epithelial cells (E-cadherin) and β-actin.
C. Cell lysates from HMLE-Snail cells treated with vehicle or 8 μg/ml PDGFRα blocking antibody were analyzed by SDS-PAGE and probed for phosphorylated PDGFRα, total PDGFRα, and β-actin.
D. Quantification of degradation in HMLE-Snail cells treated with 8 μg/ml PDGFRα blocking antibody or vehicle control. Error bars are SEM, N=3 experiments, 150 cells. * $p<0.02$.

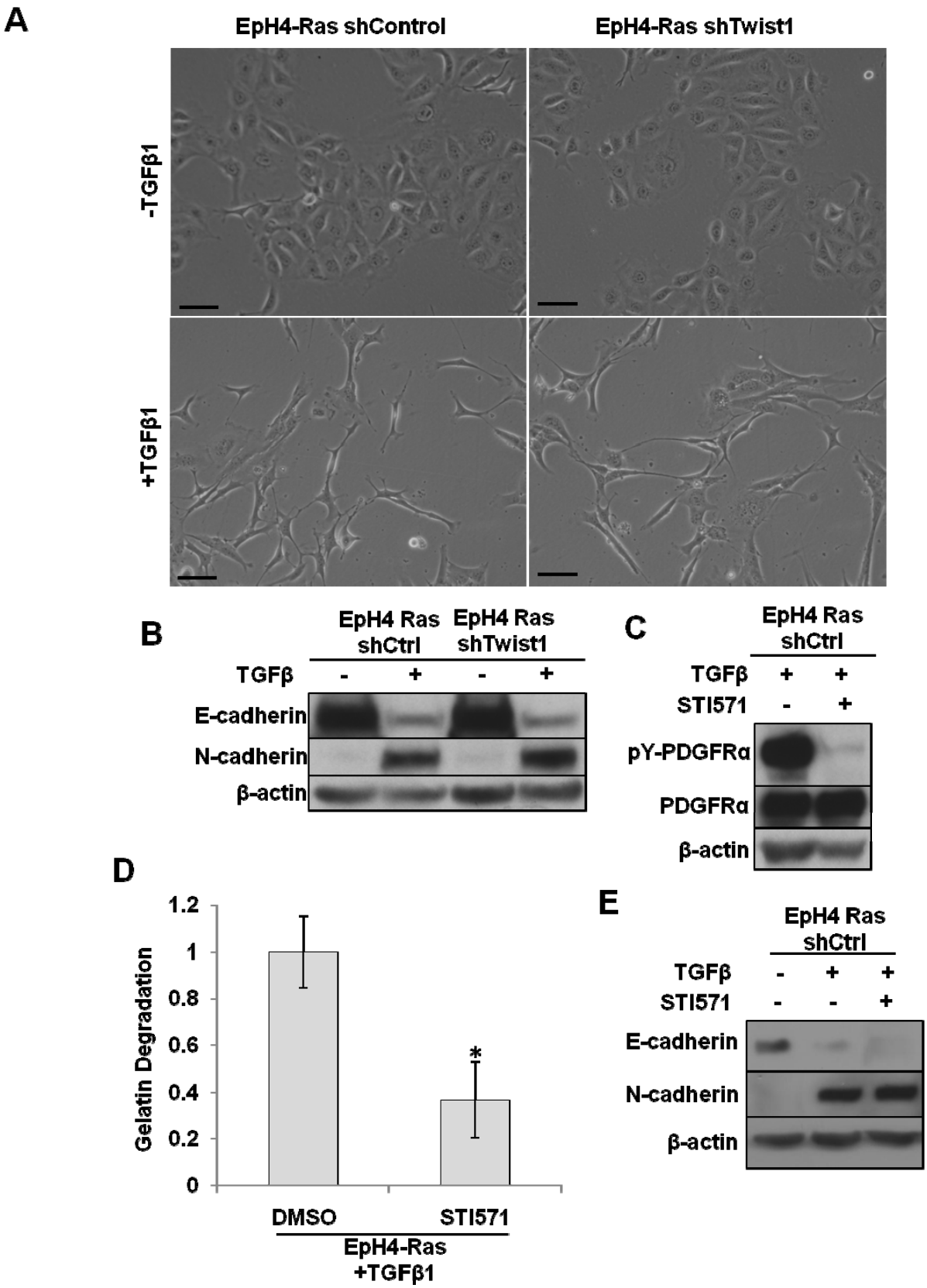

A
EpH4-Ras shControl
EpH4-Ras shTwist1
-TGFβ1
+TGFβ1
B
EpH4 Ras shCtrl
EpH4 Ras shTwist1
TGFβ - + - +
E-cadherin
N-cadherin
β-actin
C
EpH4 Ras shCtrl
TGFβ + +
STI571 - +
pY-PDGFRα
PDGFRα
β-actin
D
Gelatin Degradation
1.2
1
0.8
0.6
0.4
0.2
0
*
DMSO
STI571
EpH4-Ras +TGFβ1
E
EpH4 Ras shCtrl
TGFβ - + +
STI571 - - +
E-cadherin
N-cadherin
β-actin

Figure S6: Knockdown of Twist1 in EpH4-Ras cells does not prevent EMT and inhibition of PDGFRα suppresses matrix degradation in EpH4-Ras cells in response to TGFβ, related to figure 6.

A. Bright-field images of Eph4-Ras cells expressing control or Twist1 shRNA before and after seven days of 5 ng/ml TGFβ1 treatment. Scale bars are 10 μm.
B. Cell lysates from EpH4-Ras cells expressing control or Twist1 shRNA were collected before and after 7 days of treatment with 5 ng/ml TGFβ1 treatment, analyzed by SDS-PAGE, and probed for E-cadherin, N-cadherin, and β-actin.
C. Cell lysates from EpH4-Ras cells treated with 5 ng/ml TGFβ1 for 7 days and DMSO or 25 μg/ml STI571 for one day were analyzed by SDS-PAGE and probed for phosphorylated PDGFRα, total PDGFRα, and β-actin.
D. Quantification of degradation in Eph4-Ras cells treated with 5 ng/ml TGFβ1 and 25 μM STI571 or DMSO. Error bars are SEM, N=3 experiments, 150 cells. * $p<0.02$.
E. Cell lysates from cells with indicated treatment of TGFβ1 or STI571were analyzed by SDS-PAGE and probed for E-cadherin, N-cadherin, and β-actin.

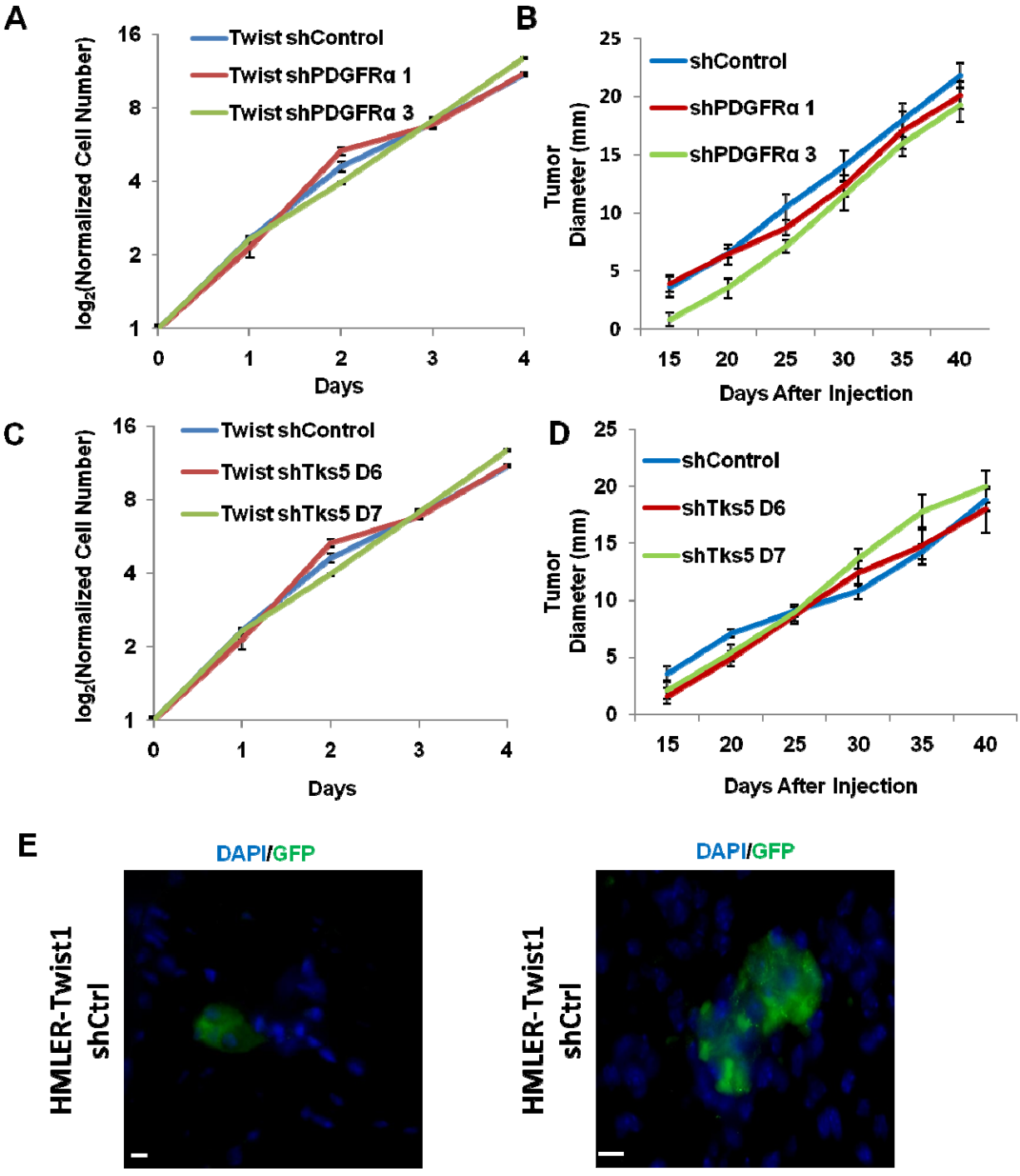

A
log2(Normalized Cell Number)
Twist shControl
Twist shPDGFRα 1
Twist shPDGFRα 3
Days
B
Tumor Diameter (mm)
shControl
shPDGFRα 1
shPDGFRα 3
Days After Injection
C
log2(Normalized Cell Number)
Twist shControl
Twist shTks5 D6
Twist shTks5 D7
Days
D
Tumor Diameter (mm)
shControl
shTks5 D6
shTks5 D7
Days After Injection
E
DAPI/GFP
HMLER-Twist1 shCtrl
DAPI/GFP
HMLER-Twist1 shCtrl

Figure S7: PDGFRα and Tks5 are not required for cell growth and primary tumor formation, but are required for metastasis to the lung, related to Figure 7.

A. HMLE-Twist1 cells expressing control or PDGFRα shRNAs were counted every 24 hours in triplicates to establish growth curve. No significant differences were observed between HMLE-Twist1 cells expressing control or PDGFRα shRNAs. Error bars are SEM.
B. Nude mice were injected with 1.5 million HMLER-Twist1 cells expressing control or PDGFRα shRNAs. Tumor diameters were measured every 5 days starting 15 days after injection until tumors reached 20 mm in diameter. N=5 mice per group. Error bars are SEM.
C. HMLE-Twist1 cells expressing control or Tks5 shRNAs were counted every 24 hours in triplicates to establish growth curve. No significant differences were observed between HMLE-Twist1 cells expressing control or Tks5 shRNAs. Error bars are SEM.
D. Nude mice were injected with 1.5 million HMLER-Twist1 cells expressing control or Tks5 shRNAs. Tumor diameters were measured every 5 days starting 15 days after injection until tumors reached 20 mm in diameter. N=5 mice per group. Error bars are SEM.
E. Representative images of GFP-positive (green) cells within lung tissues (indicated by GFP-negative, DAPI-positive cells). Cells were clustered as groups of 1-5 cells, and some larger nodules were observed occasionally. Scale bars are 5 μm.

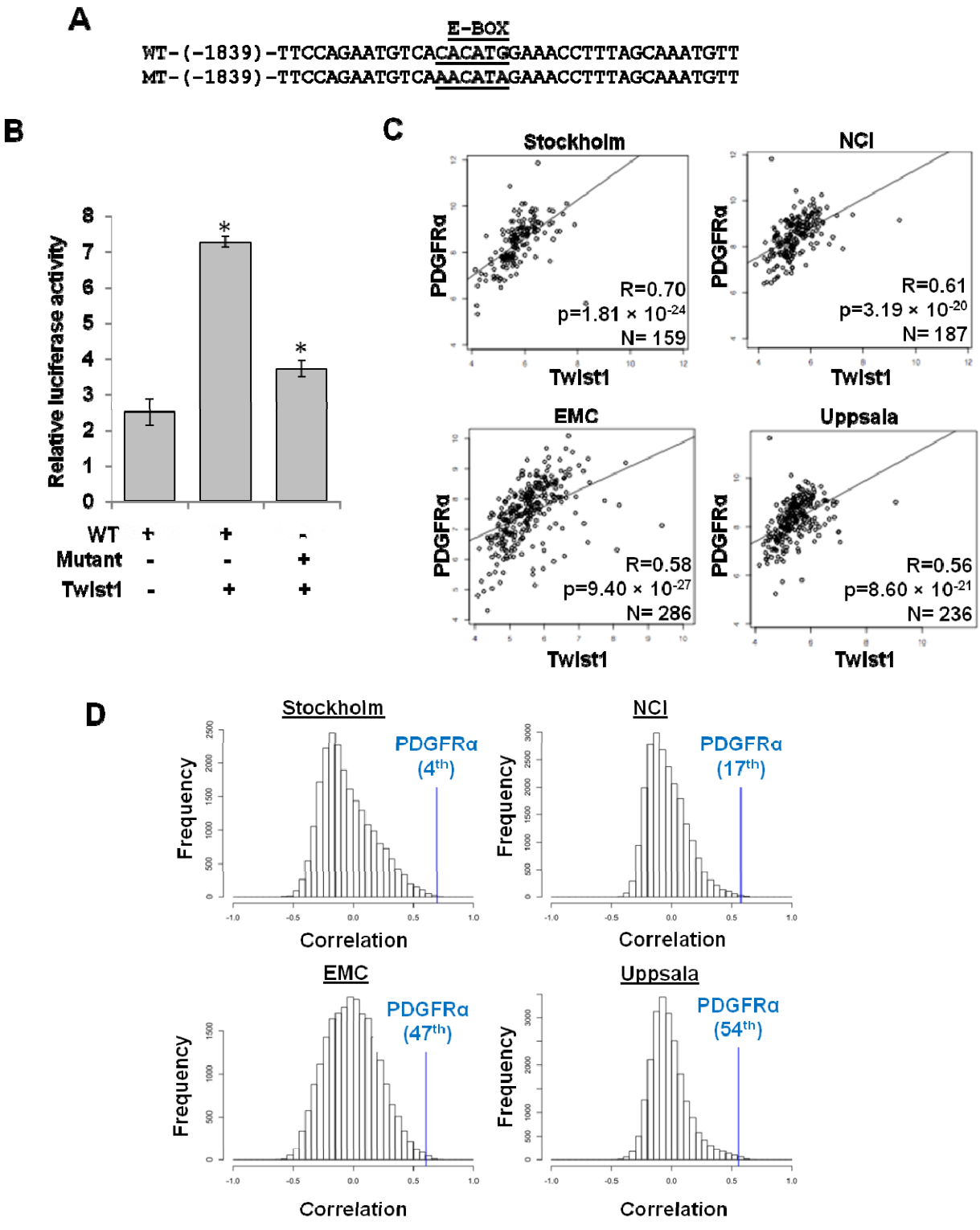

A
E-BOX
WT-(-1839)-TTCCAGAATGTCACACATGGAAACCTTTAGCAAATGTT
MT-(-1839)-TTCCAGAATGTCAAACATAGAAACCTTTAGCAAATGTT
B
Relative luciferase activity
WT + + -
Mutant - - +
Twist1 - + +
C
Stockholm
NCI
EMC
Uppsala
PDGFRα
Twist1
R=0.70
p=1.81 × 10-24
N= 159
R=0.61
p=3.19 × 10-20
N= 187
R=0.58
p=9.40 × 10-27
N= 286
R=0.56
p=8.60 × 10-21
N= 236
D
Stockholm
NCI
EMC
Uppsala
Frequency
Correlation
PDGFRα (4th)
PDGFRα (17th)
PDGFRα (47th)
PDGFRα (54th)

Figure S8: Twist1 directly induces PDGFRα transcription and expression of Twist1 and PDGFRα are correlated in breast tumor samples, related to Figure 8.

A. The E-box on the human PDGFRα promoter was mutated as indicated, WT=wild-type promoter, MT=mutated E-box-null promoter.
B. Relative luciferase activities of MCF7 cells that were transfected with indicated plasmids. N=3 experiments. Error bars are SEM. * $p<0.02$.
C. Correlation between Twist1 and PDGFRα expression in four human breast cancer gene expression datasets. Microarray measurements of PDGFRα (y-axis) are plotted against the measurements of Twist1 (x-axis). Each dot is a pair of probeset-level aggregated values for the corresponding gene. The regression line between two genes is drawn as a solid line. The Spearman correlation coefficient, p-value, and the number of patient samples in each study are listed for each dataset.
D. Histogram of ranks of correlations between all genes and Twist1 in four human breast cancer gene expression datasets. The location of the PDGFRα correlation is indicated with the blue line and the label.

Table S1: Association of PDGF ligands A, B, C, and D with Twist1 and PDGFRα, related to Figure 8.

Twist1^{HIGH}PDGFRα^{HIGH}	No Ligand^{HIGH}	Any Ligand^{HIGH}	Ligand Coexpression
Stockholm (n=51/159)	0	51	100%
EMC (n=92/286)	3	89	97%
NCI (n=69/187)	3	66	96%
Uppsala (n=76/236)	4	72	95%

Supplementary Experimental Procedures

Gelatin Zymography

Conditioned media were collected from cells at 80-90% confluency for 2 days. SDS loading buffer without DTT was added to samples of conditioned media and incubated at room temperature for 15 min. Samples were analyzed using 10% gelatin zymogram gels (Invitrogen). Gels were first incubated in Zymogram Renaturing Buffer (Invitrogen) for one hour and then incubated in Zymogram Developing Buffer (Invitrogen) overnight at 37^{o}C. Gels were stained in hot 0.1% Coomassie R-250/40% ethanol/10% acetic acid for 30 min and destained in 10% ethanol/7.5% acetic acid for 30 min.

Invasion and Migration Assays

For invasion assays, 50 μg of Matrigel was overlayed on Transwell permeable supports (Costar), dried overnight, and reconstituted with mammary epithelial growth media lacking recombinant epidermal growth factor. 40,000 cells were plated onto each well in triplicates and incubated for 72 hours. Cells were fixed with 4% PFA in PBS, washed extensively with PBS, stained with 0.1% crystal violet, washed extensively with PBS, and dried. Crystal violet was released with 50 μL 10% acetic acid and the absorbency was measured at 520 nm. Identical protocols were used for migration assays using Transwells without Matrigel. All assays were performed in triplicates.

Luciferase Reporter assay

MCF7 cells were transfected with PDGFRα prom-Luc reporter plasmid, pGL4[Rluc] plasmid, Twist1 and its dimerization partner E47. 24 hours later, the cell lysates were assayed using dual-luciferase assay kit (Promega). The firefly luciferase activity was normalized to that of Renilla luciferase to control transfection efficiency between samples.

Bioinformatics and Statistical Analysis

Four published microarray datasets were downloaded from NCBI's Gene Expression Omnibus (GEO. Obtained GEO identifiers (Study name, sample size) were GSE1456 (STOCKHOLM, n=159), GSE2034 (EMC, n=286), GSE2990 (NCI, n=187), and GSE3494 (UPPSALA, n=236). All samples were from breast cancer patients. Quantile-normalization was performed on an integrated dataset of .CEL files from individual patients to achieve the same distribution of signals across samples within a study (Bolstad et al., 2005). Multiple measurement values were aggregated at the probeset-level per patient with the Median-Polish technique (Irrizary et al., 2003). Measurement values of the two probesets were plotted in Figure S8C with a linear regression model fit. The rank-based Spearman correlation coefficient was calculated.

Spurious probesets were removed from the data analysis after a custom annotation process based on the probe sequences. In Affymetrix arrays, a gene can be represented by multiple probesets composed of 11-20 probes. However, some probe sequences do not align to an exon and therefore produced off-target measurement values (as opposed to the manufacturer's probeset-to-gene annotation). For example, none of the 11 probes of the probeset '211533_at' in HG-133A platform aligned to the gene/transcript region (chr4:54790021-54841682) of the gene PDGFRα that was the suggested target by the manufacturer's original annotation. This erroneous annotation of probesets has been previously pointed out by other investigators (Dai et al., 2005; Ferrari et al., 2007). Previously, our group has also shown that re-annotation of

microarray probes using their sequence is a crucial step in cross-platform studies of microarray data (Kuo et al., 2006). The same filtering technique was applied to the four breast cancer studies above. Once each probe was aligned to genome using BLAT (Kent et al., 2002), its genomic position was matched against AceView gene models to obtain the target gene, as AceView provides a comprehensive evidence-based gene/transcript annotation (Thierry-Mieg et al., 2006). Our selection criteria for a "good" probeset was that it needed to have all its member probes perfectly aligned within exons of a given gene using the AceView gene model. Therefore, probeset '213943_at' was chosen for TWIST1 and '203131_at' for PDGFRα. Two probesets '211533_at' and '215305_at' supposedly targeting PDGFRα in the original annotation were removed. Follow-up RT-PCR results supported our findings.

Primers

Target mRNA	Sequence
GAPDH	GAGAGACCCTCACTGCTG
	GATGGTACATGACAAGGTGC
PDGFRα	CCTGGTCTTAGGCTGTCTTCT
	GCCAGCTCACTTCACTCTCC
PDGFRβ	AGACACGGGAGAATACTTTTGC
	AGTTCCTCGGCATCATTAGGG
Twist1	TCCGCGTCCCACTAGCA
	AGTTATCCAGCTCCAGAGTCTCTAGAC
Src	GAGACGTGCTCACCATTGTG
	GCTTCTGGACGTAGTTGGCT
Fyn	TCTGCTGCCGCCTAGTAGTT
	ACAGACAGATCGGTAAGCCTT
Yes	CTCCAGAGCCTGTCAGTACAA
	CTGCTGAAATTAACTGCTGTTCC
PDGF-A	CCAGCGACTCCTGGAGATAGA
	CTTCTCGGGCACATGCTTAGT
PDGF-B	TCTCTGCTGCTACCTGCGT
	CAAAGGAGCGGATCGAGTGG
PDGF-C	ATTCACAGCCCAAGGTTTCCT
	GGGTCTTCAAGCCCAAATCTTT
PDGF-D	AAACGGCTACGTGCAGAGTC
	CCGTGTATTCTCCTGAGAGTGA

Antibodies

Antibody	Supplier	Catalog Number	WB Dilution	IF Dilution	IHC Dilution
β-actin	Abcam	ab8226	1:20000	--	--
Cortactin	Upstate	05-180	1:1000	1:1,000	--
Cortactin	Santa Cruz	sc-11408	1:2000	--	--
E-cadherin	BD Laboratories	610182	1:1000	--	--
Fyn	Alexis Biochemicals	804-564-C100	1:1000	--	--

N-cadherin	Santa Cruz	H-63	1:1000	--	--
GFP	Abcam	ab6556-25	--	1:100	--
Anti-PDGFRα	Lifespan Biosciences	LS-C11204	--	--	--
PDGFRα	Cell Signaling	3174	1:2000	--	1:200
PDGFRβ	Cell Signaling	3169	1:2000	--	--
pY^{754}PDGFRα	Cell Signaling	2992	1:2000	--	--
pY^{1009}PDGFRβ	Cell Signaling	3124	1:2000	--	--
Phosphotyrosine	Millipore	05-321	1:1000	1:500	--
Phosphotyrosine	Chemicon	AB1599	1:1000	--	--
Src	Cell Signaling	2109	1:1000	--	--
Src	Cell Signaling	2110	1:500 (IP)		
pY^{416}Src	Cell Signaling	2101	1:1000	--	--
SV40LargeT	Santa Cruz	sc-147	--	--	1:100
Tks5	S. Courtneidge	---	1:1000	1:250	--
Twist1	I. Gitelman	---	1:500	--	1:50
Yes	BD Laboratories	BD 610376	1:1000	--	--

Supplementary References

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